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ACUTE TOXICITY EFFECTS OF MERCURY AND OTHER HEAVY METALS ON HeLa CELLS AND HUMAN LYMPHOCYTES EVALUATED VIA MICROCALORIMETRY *

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SUMMARY

We have developed a microcalorimetric bioassay for acute toxicity based on heat evolution from intact cells. A reduction in heat production by cells reflects the inhibition of various biochemical reactions which constitute the cell's metabolic processes. For ten toxicants, including the heavy metals Hg, Cd, Cu, Pb, Cr, V, and As, we have measured the concentrations that are 50% effective (EC_{50}) in inhibiting heat evolution in tissue-cultured HeLa cells and in human peripheral blood lymphocytes. These data are compared with results of other acute toxicity tests and with microcalorimetric studies of heat evolution in the chemiluminescent bacterium *Photobacterium phosphoreum*. This comparison indicates that heat production may be the optimum measure of cellular metabolism for instrumental toxicological purposes since it reflects the sum of cellular metabolic processes and since it detects diffferential sensitivities of various cell types to toxicants. We have further studied effects of one heavy metal, mercury, on cell plasma membrane proteins using fluorescence photobleaching recovery (FPR) methods. Mercury induces formation of large membrane protein aggregates which disrupt cell function. FPR results suggest that, even at very low Hg concentrations, small aggregates form rapidly and increase in size causing, within one hour, measureable reductions in protein lateral diffusion.

INTRODUCTION

Chemicals in the environment represent an area of growing concern because of the possible threats of such substances to human health. Accurate toxicological data are needed to assess hazards. An acute toxicity test measures effects of toxicant concentrations on test organisms. Plots of toxicant concentration vs. effect, e.g. mortality rate or magnitude of another toxic effect, provide LC_{50} (concentration lethal to 50% of a population) or EC_{50} (concentration 50% effective in inhibition of some biological function) values. The accurate measurement of the effects of potentially toxic materials such as heavy metals and complex industrial effluents on aquatic systems depends on the reproducibility of acute toxicity tests.

Toxicity test results are limited in their applicability because they are difficult to reproduce. Toxicities of substances vary depending on the chemical conditions of the test (ref. 1) and on the variability inherent in biological samples (ref. 2). A toxicity test should be suited to studies using various animal species of interest and should measure a physiological parameter

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that can be electronically detected. Among the parameters that have been used as indicators of cellular metabolism in toxicological contexts are carbon dioxide production (ref. 3) and chemiluminescence (ref. 4). Calorimetry has been particularly useful in monitoring cellular metabolism and heat measurements have long been used to study metabolism in cells and whole organisms (ref. 5). Experiments comparing heat production to oxygen consumption and to evolution both of CO_2 and of light have been performed (refs. 6-7).

Calorimetry of living biological systems is a well-established field. Lamprecht *et al.* examined yeast metabolism (ref. 8) via microcalorimetry. Beezer *et al.* demonstrated that microcalorimetric studies of bacterial growth show details in growth curves not observable by other techniques (ref. 9). Calorimetric measurements were employed by Fortier *et al.* to measure biodegradation processes in wastewater (ref. 10). Belaich *et al.* measured calorimetrically the effects of external glucose concentration on the metabolic activity of anaerobically growing cells (ref. 11). Binford's microcalorimetric method of antibiotic sensitivity testing using fifteen different strains of clinically important bacteria showed that therapeutically appropriate antibiotics could be successfully selected by this method (ref. 12). Arhammer *et al.* examined calorimetrically the kinetics of antibiotic action on several strains of bacteria (ref. 13). Beezer studied effects of disinfectants (ref. 14) and antibiotics (ref. 15) on bacterial cultures under various conditions. The inhibition of metabolism in yeast cells by antibiotics was higher at low pH and decreased as the pH was raised toward neutral. Effects of antibiotics were also decreased in the presence of calcium and magnesium ions. Jolicoeur *et al.* used flow microcalorimetry to investigate the effects of aliphatic alcohols of various chain length on heterogeneous bacterial cultures (ref. 16). Beaubien *et al.* used flow microcalorimetry to observe the effects of various surfactants on aerobically growing cells (ref. 17). Schon and Wadsö measured the effects of antineoplastic drugs on mammalian cells (ref.18). Wadsö *et al.* also measured calorimetrically the heat production in human skeletal muscle under various conditions (ref. 19).

Various investigators in this field have correlated microcalorimetric measurements of cellular metabolic rates with cellular functions. For example, metabolism in various cell types during phagocytosis was measured calorimetrically. Rialdi and Eftimiadi (ref. 20), by measuring O_2 consumption and CO_2 and lactic acid production, calculated the theoretical enthalpy change per mole of glucose oxidized and found this value to agree with the actual measured heat production. Rialdi *et al.* performed several quantitative calorimetric studies of protein binding such as concanavalin A binding to manganese, saccharides, and immunoglobulins (ref. 21). Wadsö *et al.* used calorimetry to detect antigen-antibody complexes by measuring heat production in blood lymphocytes as specific amounts of antigens and antisera were introduced

(ref. 22). Further studies showed that various immune complexes induce heat production by binding either to $F(ab)_2$ fragments in the presence of complement or to Fc fragments (ref. 23). Schon and Wadsö measured heat production rates along with CO_2 and lactate production rates in Vero cells and correlated heat production both to amounts of protein consumed in cell samples and to cell number (ref. 24). Monti *et al.* measured heat production in lymphoma cells and found it to decrease as patients responded to treatment and associated high heat production in these cells with poor prognoses (ref. 25). These results are consistent with calorimetric measurements on fat cells from obese subjects which showed lowered metabolic activity in these cells after weight reduction (ref. 26). The good reproducibility of calorimetric measurements on cellular systems has been demonstrated in a number of studies on heat production in human lymphocytes in optimal media at 37°C. Monti *et al.* (ref. 25) report 3-5 pW per cell, Levin (ref. 27) reports 5.9 ± 1.6 pW per cell, and Krakauer and Krakauer report 3.5 pW per cell (ref. 28).

The current maturity of cellular calorimetry motivated our development of a microcalorimetric acute toxicity assay. We have measured EC_{50} values based on heat production in HeLa cells for ten toxicants including the heavy metals Hg, Cd, Cu, Pb, Cr, V, and As. Microcalorimetry, however, being a non-specific indicator of toxicant interference with cellular metabolism, does not provide information on the mechanisms of toxicity. Among significant pollutants, the heavy metals are those whose mechanism of action on cells can best be studied. While there is considerable information on the toxic effects of these metals at or higher than their EC_{50} concentrations (refs. 29-34), little is known about their effects at the cellular level at lower concentrations. It is generally believed that toxic effects of heavy metals arise from metal-induced crosslinking and aggregation of plasma membrane proteins. Membrane proteins have important cellular functions, serving as ligand receptors, ion channels and nutrient transporters. Formation of protein aggregates may thus seriously compromise cellular function. However, at this time, concentration- and time-dependent effects of these metals on membrane proteins have not been evaluated and it is not known to what extent small degrees of crosslinking of plasma membrane proteins may disrupt cellular functions. In this study, we have examined the effects of one heavy metal, mercury, on plasma membrane proteins. We have used fluorescence photobleaching recovery (FPR) to examine the cell surface events caused by exposure to acutely toxic concentrations of mercury. FPR is a technique that measures movements of specific fluorescently labeled molecules in cell membranes. An argon ion laser is focussed on a micrometer-sized area of the cell membrane and fluorescence from the region is monitored over time. A brief pulse of more intense light bleaches fluorophores in this area. Subsequent fluorescence recovery kinetics reflect the diffusion of new fluorophores into the

bleached region. An excellent discussion of this technique has been provided by Ware (ref. 35).

METHODS

Buffers and Stock Solutions

HEPES buffer (0.025 M; Sigma Biochemical, St. Louis, MO) was prepared using double-distilled water and the pH adjusted to 7.2. Piperazine N,N-bis(2-ethanesulfonic acid) (PIPES; U.S. Biochemical Corporation Cleveland, OH) was used as a buffer for toxicant solutions. Buffer solutions 0.05 M in PIPES were prepared using 1.0 M sodium hydroxide to dissolve the free acid in distilled water. Stock toxicant solutions of potassium cyanide (Fisher Scientific Co., Fair Lawn, NJ), mercuric chloride (Mallinckrodt Chemical, St. Louis, MO), cadmium chloride (J.T. Baker Chemical Co., Phillipsburg, NJ), cupric chloride (Baker), lead acetate (Baker), acetone (Fisher), phenol (Fisher), toluene (Mallinckrodt), ammonium metavanadate (Aldrich Chemical Co., Milwaukee, WI), vanadyl sulfate hydrate (Aldrich), and the commercial wood treatment formulation CCA composed of 7.4×10^{-3} M arsenic (V) oxide, 1.16×10^{-2} M copper (II) oxide, and 2.35×10^{-2} M chromium (III) oxide (Simonsen Chemical Co., Cabool, MO) were prepared at concentrations in the range of published toxicity values. All chemicals were analytical grade and double distilled water was used to prepare all solutions.

HeLa Cells and Cell Culture

The S3G strain of HeLa cells, a human epitheloid carcinoma, was purchased from American Type Culture Collection (Rockville, MD). Growth medium for HeLa cells was Eagle's minimal essential medium with non-essential amino acids and Earle's balanced salt solution, 1% L-Glutamine and 1% penicillin-streptomycin from Sigma Biochemical (St. Louis, MO), and 10% sterile filtered supplemented calf serum or fetal bovine serum from Irvine Scientific (Irvine, CA). Trypsin for dislodging adherent cells from tissue culture flasks was purchased from Sigma Biochemical. Solutions of 0.25% trypsin in 0.9% sodium chloride were prepared and sterilized using Nalgene 0.22 μm sterile filters (Nalge Co., Rochester, NY). HeLa cells were grown in the culture medium described previously and maintained in a Forma Model 3157 incubator at 37°C. Trypsin was used to dislodge the cells from the surface of the flask. Suspended cells were then placed in sterile centrifuge tubes and centrifuged for five minutes at 1000 rpm. The cells were washed twice with growth medium. Doubling time for cell cultures was 2.5 days. Counts of viable cells were performed using trypan blue exclusion before introducing samples into the calorimeter. Fifty μL aliquots of cell suspensions were diluted to 100 μL in trypan blue solution (0.2 weight % in BSS) and examined in a hemacytometer (ref. 36). Lyophilized samples of Microtox bacteria were donated by A. Bulich at Beckman Inc.

(Carlsbad, CA).

Microcalorimetric Toxicity Measurements

An LKB Model 10700-1 heat conduction flow microcalorimeter mounted in a Tronac PTC-40 temperature controlled water bath was used to measure metabolic heat production in cells treated with toxicants. The detection limit of the calorimeter is 0.1 microcalorie per second or 0.4 microwatt as specified by the manufacturer. For the calorimetric toxicity assay the cells, medium and toxicant are mixed outside the calorimeter and then pumped into the flow-through sample cell. Medium containing no cells is pumped into the mixing reaction cell. Cellular metabolism then raises the sample temperature by a steady-state value proportional to the rate of cellular heat production. The voltage difference between the thermopiles, proportional to the rate of heat evolution in the cell suspension, is amplified by a Keithley Model 150B microvolt ammeter and recorded on a strip chart recorder. Calibration is performed by applying a known electrical current to a calibration heater and the sample heat evolution in watts per cell is calculated. From these raw data, heat production by cells in the presence and absence of toxicant, toxicity of the test substance at its known concentration is evaluated.

Cells were suspended in 3.0 mL of fresh medium to provide a constant source of energy throughout the experiment. They were pumped into the calorimeter with an LKB Perpex peristaltic pump at a flow rate of 0.5 mL per minute. Cell concentrations ranged from 8×10^5 to 2×10^6 cells/mL. Samples of suspended cells were gently agitated during pumping to maintain homogeneity in the number of cells pumped into the calorimeter flow cell. The initial calorimetric response to injection of sample cells is a voltage signal arising from the viscous heat of fluid flow through the sample cell. When the pump is turned off the signal decays to a steady-state heat output corresponding to the metabolic heat production in the cells. Heat evolution was monitored for at least thirty minutes. Heat production in HeLa cells ranged from 25-50 pW per cell. Over the course of experiments cells remained >90% viable as shown by fluorescein diacetate viability assays.

Experiments were conducted with toxicant solutions in the pH range of 6.8 to 7.4. All cells were suspended in medium containing HEPES buffer. This minimizes changes in pH arising from cellular CO_2 production. Concentrations of toxicant solutions were chosen based on published acute toxicity values. PIPES buffer was used for all stock toxicant solutions because it has low metal binding coefficients and a pK_a of 6.8. Fixed amounts of toxicant solutions of known concentrations were added to cell samples immediately before introduction into the calorimeter to make a total volume of 3.0 mL.

Heat production in cell samples containing toxicants rarely reached a steady state; the

cells' metabolic rates generally decreased as a function of exposure time to toxicants. All measurements of cellular toxicity therefore were made at thirty minutes' exposure time. To eliminate the variability introduced as a result of culture conditions, heat production in cell samples containing toxicants was compared with that in control cell samples harvested on the same day.

Toxicity Studies Using Human Lymphocytes

Lymphocyte-enriched human cell populations were prepared from whole blood donated by healthy adult volunteers via centrifugation over Histopaque (Sigma Biochemical). 20 mL samples of whole blood were diluted 2x in BSS. 10 mL of Histopaque separating solution were added to each of four centrifuge tubes and 10 mL of diluted blood then carefully layered over the Histopaque. Samples were centrifuged at 3000 rpm for 30 minutes at 22°C. White layers of mononuclear cells were removed into two clean centrifuge tubes and washed 3 times in BSS. These preparations typically contain about 10% macrophages (ref. 36). Cells were then resuspended in minimal essential medium and small aliquots were taken for cell counts. Toxicants were added and suspensions made up to 2.0 mL with medium. Toxicant concentrations were within the ranges of microcalorimetric EC_{50} values for HeLa cells and chosen arbitrarily. These experiments were performed at 37°C. The rate of heat production in these cells ranged from 1.0 to 3.0 pW per cell. Non-phagocytosing macrophages produce about 3.7 pW/cell under our experimental conditions (ref. 36a) and therefore make a relatively minor contribution to the metabolic heats measured. Data were processed as described in Results.

Fluorescence Photobleaching Recovery Measurements

Cells (10^6 cells/mL) were washed twice and incubated in balanced salt solution (BSS) containing toxicants for varying amounts of time. The cells were then washed once and resuspended in 1 mL of BSS. The viability of HeLa cells was always greater that 95% as judged by trypan blue dye exclusion. Fluorescence photobleaching recovery measurements on tetramethylrhodamine (TRITC)-succinyl concanavalin A (SConA) labeled cells were performed at 37°C under coverslip on well slides using the FPR system described previously (ref. 37). Healthy, viable cells are selected microscopically for each measurement; a single data point represents between 15 and 25 cells on each of which a single measurement was made. Enlarged cells, cells with damaged membranes, membrane blebs or other features typical of dead or damaged cells were not used in these experiments. Molecules labeled with TRITC-SConA within a less than 1 μm^2 beam region were bleached by a 100 msec pulse of 3 mW, 514.5 nm

light. The laser beam intensity was then attenuated 30,000 fold, and fluorescence from the bleached area was measured by a thermionically cooled C31034A photomultiplier tube operated in a single photon counting mode. A photobleaching experiment on an individual cell consisted of 128 fluorescence measurements at 20 msec/point to establish the prebleach fluorescence, a 100 msec bleaching pulse, and at least 240 additional 20 msec fluorescence measurements to delineate the fluorescence recovery kinetics. Data were analyzed on-line by using optimized nonlinear curve fitting procedures to obtain the diffusion coefficient and the mobile fraction of the fluorescent molecules being studied.

RESULTS

Calorimetric Toxicity Measurements

The substances used in our microcalorimetric toxicity studies, lead, copper, cadmium, mercury, vanadium, chromated copper arsenate (CCA), acetone, phenol, toluene, and cyanide, are materials whose acute toxicity has previously been measured by various methods. CCA was

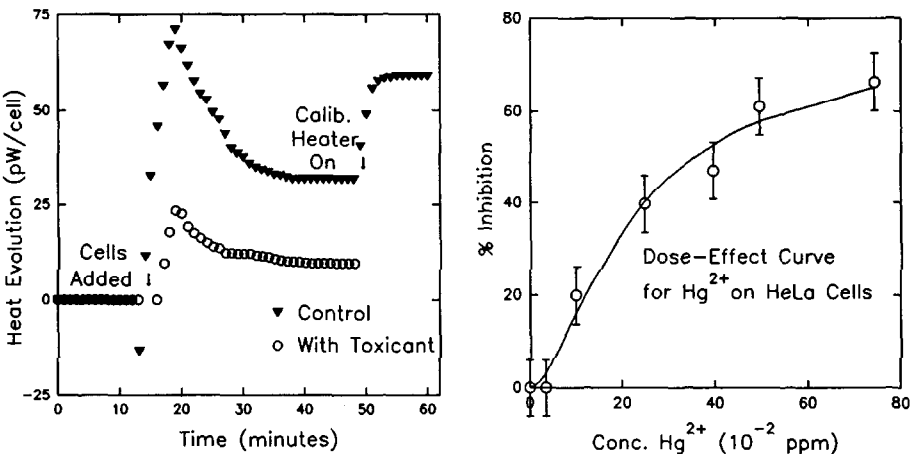

Figure 1 (left). Raw data showing heat production in HeLa Cells exposed to chromated copper arsenate (CCA). Test sample (o) contains 1.5 x 10^6 HeLa cells and 800 ppm CCA. The control (▾) contains 1.5 x 10^6 HeLa cells and no toxicant. The calibration heater power is 40.5 μW. Figure 2 (right). Dose-response curve for inhibition of heat production in HeLa cells exposed to Hg^{2+} at 37°C.

chosen as a typical mixture of toxicants generally representative of environmental samples. Figure 1 illustrates the type of raw data obtained from HeLa cells alone ("control") and from HeLa cells treated with CCA ("with toxicant"). The steady-state heat evolution at thirty minutes is 31.8 pW/cell for the control and 9.6 pW/cell for cells treated with CCA. The calorimetric

toxicity is expressed as percent inhibition of heat production in cells exposed to the toxicant relative to the control sample. Percent inhibition is defined as

$$\%I = 100 \times (P_c - P_t) / P_c \quad (1)$$

where %I is the percent inhibition of heat production, P_c is the rate of heat production in the control, and P_t is the rate of heat production in the toxicant-treated sample. In Figure 2, %I = 70%. The EC_{50} for a toxicant is the concentration that produces 50% inhibition of heat production and is interpolated from a dose-effect curve of %I vs. toxicant concentration. EC_{50} values for the toxicants studied are listed in Table I.

Table I: Comparison between Rainbow Trout LC_{50}, E. coli 40% I, Microtox Chemiluminescence EC_{50}, and Microcalorimetric EC_{50} values for various toxic substances

Toxicant	Rainbow Trout LC_{50} (ppm)[a]	E. coli Electrode 40% I (ppm)[b]	Microtox Chemi-luminescence EC_{50} (ppm)[c]	Micro-calorimetry HeLa cells EC_{50} (ppm)[d]	Micro-calorimetry Microtox EC_{50} (ppm)[e]
Cadmium	0.006	0.3	21.0	14.4	~21.0
Copper	0.4	1.1	8.0	1.3	< 8.0
Lead	1.2	0.13	5.0	0.32	< 5.0
Mercury	0.05	0.3	0.046	0.26	> 0.05
Cyanide	0.7	4.0	4.8	16.1	~ 5.0
Toluene	44.0	N.D.	50.0	3.3	< 50.0
Phenol	46.0	1209.0	28.0	180.0	N.D.
CCA	N.D.	0.01	N.D.	0.04	N.D.
Vanadium (IV)	N.D.	N.D.	N.D.	0.01	N.D.
Vanadium (V)	N.D.	N.D.	N.D.	0.35	N.D.

[a] LC_{50} values are obtained from 48 or 96-hour exposures. References are as follows: cadmium, copper, lead, cyanide, and phenol (ref. 3), mercury and toluene (ref. 9).
[b] 40% I values for all toxicants obtained from reference (ref. 39).
[c] EC_{50} values were obtained from 15-minute exposures at 15°C test temperature. References are as follows: cadmium, lead, cyanide, and phenol (ref. 3), mercury and copper (ref. 41), toluene (ref. 42).
[d] EC_{50} values were obtained by interpolation of dose-response curves.
[e] EC_{50} values were estimated from measurements of heat production in Microtox bacteria in the presence of Microtox EC_{50} concentrations of toxicants.

An example of calorimetric toxicity data is presented in Figure 2. The graph is a dose-effect curve for mercury. The dose in these experiments is the total concentration of toxicant present in the medium in which the HeLa cells are suspended. The effect being measured is the percent inhibition of heat production in suspended cells after thirty minutes' exposure to a particular concentration of some toxicant. Heat production in control samples represents zero percent inhibition. All dose-effect data were processed by non-linear curve

fitting. Each measurement was made using a different cell population. Mercury exhibited a small concentration range over which toxic effects in HeLa cells could be measured. At 0.042 ppm, mercury caused 18% inhibition of cellular heat production. Metabolic inhibition reached a plateau of approximately 70% there being no appreciable increase in effect with additional toxicant between 0.49 ppm and 0.75 ppm. All measurements of toxicity were carried to high toxicant concentrations relative to the effective concentration range to determine if dose-effect plots were multiphasic. Multiphasic plots suggest multiple target sites in cells having different affinities for the toxicant. In the case of mercury, maximum inhibition of heat production was approximately 70%. The calorimetric EC_{50} of mercury interpolated from the dose-effect curve is 0.26 ppm. The dose-effect curves for the other toxicants are similar to that shown for mercury with generally larger ranges of effective concentration. Some dose-effect curves exhibited concentration thresholds, well known in acute toxicity studies, below which no toxic effects were observable and above which toxic effects occur. The shape of a dose-effect curve is partially a function of exposure time. We observed a wide range of EC_{50} values for the toxicants studied. Table I lists data comparing calorimetric thirty minute EC_{50} values with rainbow trout 48-96 hour LC_{50} values, *E. coli* electrode 40% inhibition values, and Microtox five to fifteen minute EC_{50} values.

Similar results were obtained using lymphocyte-enriched human cell populations. Figure 3 shows the voltage signal from a sample of lymphocytes exposed to 0.02 ppm Hg^{2+}, a concentration approximately 10x lower than its HeLa calorimetric EC_{50}, for thirty minutes. The steady state energy output from the control sample was 0.77 pW/cell and that from the sample with mercury was 0.53 pW/cell. This represents 32% inhibition of heat production. Results

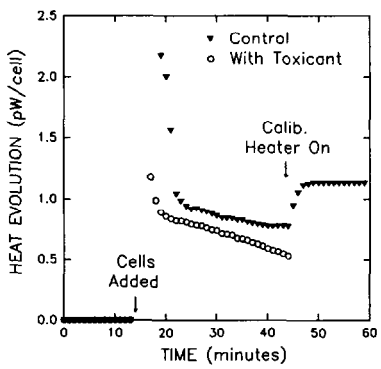

Figure 3. Raw data showing heat production at 37°C in human peripheral blood lymphocytes exposed to Hg^{2+}. Test sample (o) contains 2.2 x 10^7 lymphocytes and 0.02 ppm Hg^{2+}. The control (▾) contains 4.0 x 10^7 lymphocytes and no toxicant. The calibration heater power is 8.0 μW.

TABLE II
Calorimetric Toxicity Measurements Using Human Peripheral Blood Lymphocytes

Toxicant	Conc (ppm)	pW/cell (toxicant)	pW/cell (control)	%I
Hg^{2+}	0.02	0.53	0.77	32
	0.05	1.4	1.9	26
	0.08	0.5	1.7	71
CN^-	7.6	0.0	3.2	100
VO_3^-	0.02	0.0	1.34	100

of microcalorimetric measurements on lymphocytes are listed in Table II. Lymphocytes appear to be more sensitive to toxicants than HeLa cells. When exposed to concentrations of cyanide and vanadium (V) approximately 2-fold and 100-fold lower, respectively, than their HeLa calorimetric EC_{50} values, lymphocyte heat production was 100% inhibited. Moreover, lymphocytes appear to be more sensitive to small changes in toxicant concentration than HeLa cells. For example inhibition of heat production increased from 32% to 71% over a 0.06 ppm range.

FPR Measurements Using HeLa Cells

We used fluorescence photobleaching recovery techniques to determine whether exposure to mercury at its EC_{50} has a significant effect on the lateral diffusion of HeLa cell plasma membrane glycoproteins (Table III). HeLa cells were treated with the indicated concentrations of Hg^{2+} prior to labeling with 10 μg/mL TRITC-SConA. Concanavalin A is a plant lectin that

TABLE III
FPR Measurements of TRITC-SCon A labeled glycoprotein lateral diffusion on Hg-treated HeLa Cells

Hg^{+2} Conc (ppm)	Time (hr)	Diff Coeff ($10^{-10}cm^2sec^{-1}$)	% Recovery
0	0	10.5± 9.3	52.7 ± 6.6
.24	1.0	5.7 ± 0.6	55.3 ± 5.8
1.0	1.0	4.8 ± 2.3	54.7 ± 5.0
2.2	1.0	not measureable	< 20
20.0	1.0	not measureable	< 20
.24	4.0	3.9 ± 1.5	49.9 ± 7.1
.24	7.0	2.8 ± 0.4	44.3 ± 6.3
.24	10.0	not measureable	< 20

binds glucose and mannose residues on membrane glycoproteins and that has been extensively use to probe the physical motions of glycoproteins on various cell types. One hour exposure to 0.24 ppm Hg^{2+} causes an almost 50% reduction in glycoprotein lateral diffusion, a result also obtained with a 4-fold higher Hg^{2+} concentration and readily explained by Hg^{2+}-induced formation of moderately large protein aggregates. Exposure of HeLa cells to 2.2 ppm Hg^{2+} reduced the fraction of laterally mobile proteins to less than 20%, indicating that only very large, slowly diffusing protein aggregates were present on the cell membrane. Longer exposure to Hg at 0.24 ppm had a similar effect. Protein lateral diffusion slowed to $2.8 \times 10^{-10} cm^2 sec^{-1}$ after a seven hour treatment. By 10 hr, membrane complexes are sufficiently large to have diffusion coefficients unmeasurable by FPR methods ($< 10^{-12} cm^2 sec^{-1}$).

DISCUSSION

For each toxicant studied, percent inhibition of heat production increased with toxicant concentration. All the dose-effect curves had hyperbolic shapes with relatively steep initial slopes and with plateaus achieved at characteristic concentrations. The specific shapes of the dose-effect curves and the ranges of effective toxicant concentration varied. Factors which determine the characteristics of a dose-effect curve are the toxicant's mode of action in cells, its number of target sites and its affinity for those target sites. The toxicity of a metal to cells depends on its oxidation state, speciation, and the stability and solubility of its compounds. Highly soluble compounds of mercury, lead, copper and cadmium were chosen. The microcalorimetric studies showed a correlation between toxicity and sulfhydryl affinity suggesting that cross-linking membrane proteins is a major factor in the toxic effects of these metals. The relative affinities of the metals for sulfhydryl groups, Hg > Pb > Cu > Cd (ref. 38), are in agreement with the relative toxicity values. The EC_{50} for mercury in HeLa cells is 50 times lower than that of cadmium. Mercury, having the highest sulfhydryl affinity, produced its maximum metabolic inhibition in HeLa cells at 0.5-0.75 ppm. Cadmium produced its maximum toxic effect at 20-30 ppm.

As seen in Table I, microcalorimetric data accord well, for the most part, with those from the other toxicity tests, namely rainbow trout 96 hr. mortality (refs. 3,9), the *E. coli* electrode (ref. 39), and the Microtox chemiluminescence assay (refs. 3,41,42). Variations in effects of toxicants are to be expected when different cell species and whole animals are used as target organisms. Data analyses for the three cellular tests are similar. In all cases percent inhibition of metabolic functions are measured: light production in the Microtox system, CO_2 production in the *E. coli* electrode system, and heat production in the microcalorimetric system. Still, the EC_{50} values for the toxicants used in this research differ because each bioassay uses

a different cell type and monitors a different metabolic function.

Since our microcalorimetric method is adaptable to arbitrary cell types, we were able to compare metabolic effects of toxicants on HeLa cells and on the Microtox bacterium *Photobacterium phosphoreum*. We were also able to compare *P. phosphoreum* toxicity data based on its heat production to that based on its chemiluminescence. We measured calorimetrically the effects of six toxicants on heat evolution by *P. phosphoreum* for Microtox bacteria when the toxicants were present at specific concentrations. These concentrations were the EC_{50} concentrations determined from chemiluminescence inhibition in the commercial Microtox bacterial assay. EC_{50} values based on heat production in Microtox bacteria were in better agreement with results from other assays than the EC_{50} values based on chemiluminescence. Heat evolution reflects all energy-producing pathways in the cell whereas chemiluminescence is the product of only one specific pathway. Calorimetric studies of cellular oxidative metabolism in human neutrophils showed that light production represents only a fraction of the total metabolic energy in these cells (ref. 7). Therefore, as our results indicate, heat production may be a better predictor of organismal toxicity than chemiluminescence because heat production directly reflects overall metabolism.

One hypothesis motivating this work was that toxicity results based on living cells of a particular species would have special value relative to tests based on whole animals of another species or on prokaryotic organisms. The experiments using lymphocyte-enriched human cell populations illustrate that different species indeed have different sensitivities to toxicants and that calorimetry is capable of measuring these differences.

The microcalorimetric system for measuring acute toxicity of substances in water thus fulfills the requirements for an effective instrumental acute toxicity test. It can measure toxicities of substances regardless of their mechanisms of toxic action. It can be used to study water samples that are colored or turbid. Instrumental responses are reproducible and the system is inexpensive and easy to use. The microcalorimetric toxicity assay is suited to studies on cells of any species. In summary, our studies of the microcalorimetric toxicity assay have demonstrated considerable potential utility of microcalorimetry in future toxicological research.

Our FPR results suggest that the primary effect of heavy metals on cells is to crosslink plasma membrane proteins. Preliminary experiments with FPR have demonstrated that HeLa cell exposure to mercury at concentrations near its EC_{50} causes large decreases in glycoprotein lateral diffusion. FPR results are particularly significant given the fact that they reflect only large changes in protein aggregate size. Thus, these results suggest that small protein aggregates rapidly form in cells treated with Hg^{2+} and increase sufficiently in size by 1 hr to cause measurable reductions in protein lateral diffusion. Because aggregation has profound effects on

protein function (ref. 40), *any* observable formation of protein aggregates by metals must be of physiological significance. We expect that more sensitive methods will demonstrate significant membrane restructuring by metal concentrations now considered inconsequential. Such observations would motivate a reexamination of what constitute "acutely toxic" levels of aquatic pollutants. This is a particularly critical point since current U.S regulations establish safe levels of aquatic pollutants at concentrations 1/10th that of their EC_{50} (ref. 43).

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