In Vitro Cellular Uptake of Benzo [*a*] pyrene Measured by a Microfluorometric Technique[1] (35423)

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Investigations of early interactions between chemical carcinogens and cells in culture are providing new insight into events preceding neoplastic transformation (1–6). These carcinogens are toxic (7–12) and can induce in certain cells an aryl hydrocarbon hydroxylase (AHH) (13–15) that may be linked to both toxicity and transformation (16–17). All of these investigations have involved *populations* of normal cells; whereas few, if any, studies have examined *quantitatively* the interaction between chemical carcinogens and *single* cells. We have developed a new microfluorometric technique for quantitating the uptake of benzo[a]pyrene (BP) into individual cells in culture (18). This detailed report of our observations shows that (a) in cultured mammalian cells there is a correlation between BP uptake and BP-induced cytotoxicity, and (b) at nonlethal BP concentrations, the cell can rid itself to some extent of this chemical carcinogen.

Materials and Methods. Cell line. The cell line used throughout this work was the V-79-4 line[4] derived from lung tissue of the Chinese hamster. Cells were maintained in Eagle's minimal essential medium supplemented with 15% fetal calf serum (FCS) plus antibiotics (100 units of penicillin/ml and 100 μg of streptomycin/ml). All cultures were maintained in darkness and examined under strict light conditions which excluded ultraviolet and short wavelength visible light.

BP stock solutions. Ten mg of BP[5] were dissolved in 2.0 ml of *N,N*-dimethylformamide (DMF) and this solution was added to 19 ml of FCS. The resulting suspension was stirred for 24 hr at 37° and undissolved BP removed by two 30-min centrifugation runs at 5000*g* at 4°. The decantate was stored at 4° for no more than 48 hr before use. The amount of BP dissolved in 1.0-ml samples of FCS was determined by extraction with 10 ml of heptane for 48 hr at 37°. The BP in the heptane phase was measured fluorometrically in comparison with standard heptane solutions of BP (19). Measurements of the remaining BP fluorescence of the FCS indicated that 95–99% of the BP had been extracted. BP solubility in FCS was found to be 24 to 35 μg/ml. For treatment of cells, media were made up to a total of 15–20% FCS with BP-FCS and regular FCS, depending on concentration of BP desired. FCS which had been stirred, centrifuged, and stored as above but containing no BP was used in control media for toxicity experiments.

Fluorescence assay. Excitation light for BP assay was the 366-nm line from a 200-W high pressure mercury arc lamp. Isolation of this wavelength was accomplished with a Bausch and Lomb model 33-86-25-05 monochromator plus two CS-7-54 ultraviolet transmitting filters[6] and a CS-1-69 heat filter.[6] Fluorescence was measured using a Leitz ortholux fluorescence microscope equipped with Ploem (20) illuminator, barrier filters, MPV

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photometer[7] and 60 mm interference wedge filter.[7] For BP quantitation we isolated the BP emission band which peaks at 454 nm. This was done by selection of the 400-nm dichroic mirror in the Ploem illuminator and a 430-nm barrier filter, and by positioning the interference wedge for transmission in the region 420–480 nm. Intensity of BP emission at 454 nm was measured with an EMI-6094 photomultiplier using a model 414 micromicroammeter.[8] Fluorescence assay for possible presence of 3-hydroxybenzo[a]pyrene (3-OH BP) or related metabolic products (21) of BP was carried out as above except that (a) excitation light of 400-nm wavelength was isolated by the monochromator in combination with a heat filter, and (b) emission of 3-OH BP which in alkaline solution peaks at 522 nm was distinguished from BP fluorescence by use of a 450-nm dichroic mirror in the Ploem illuminator and 430-nm barrier filter and by positioning the interference wedge filter for transmission in the region 490–550 nm. Corrections for day-to-day variations in sensitivity were made using quinine sulfate (blue) and uranium glass (CS-3-79)[6] (green) as fluorescent standards. When measuring fluorescence of solutions in 1.0-mm cuvettes, the 25× objective of the microfluorometer was placed in contact with the cuvette. Fluorescence of solutions in the hemocytometer was measured using the 25× objective and a variable rectangular measuring diaphragm in the MPV photometer. In this way, we superimposed the image of one square of the hemocytomer over this diaphragm and thus measured fluorescence from a known volume of liquid having a known BP concentration. Figure 1 shows that the microfluorometer response is linear over several orders of magnitude of concentration of fluorescent material under a variety of conditions. We could thus obtain values of photomultiplier current in units (A/g of BP). This number was multiplied by 1.5 to correct the calibration for out-of-focus effects in the upper regions of our hemocytometer.

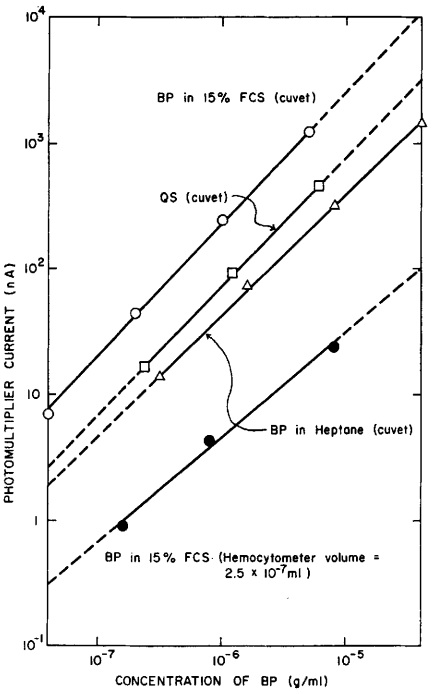

FIG. 1. Standard fluorescence curves of benzo(a)pyrene (BP) and quinine sulfate (QS) in terms of photomultiplier current. Measurements of liquids in cuvettes were made with the microfluorometer measuring diaphragm wide open.

Since the cellular fluorescence measurements were made using the same setup, values of photomultiplier current per cell could be divided by this standard value of 3.6×10^4 A/g of BP (in FCS) to give the amount of BP per cell. A similar calibration for quantitating 3-OH BP[9] was not made since cellular fluorescence was measured using the 70× rather than the 25× objective.

Measurements of cellular uptake of BP. The kinetics of BP uptake were monitored in two ways—fluorescence assay and liquid scintillation detection of tritiated BP. For both studies, cells were grown on coverslips in 60-mm plastic petri dishes.[10] These coverslips were then placed in sterile Columbia jars containing BP-media [for radioisotope detection, medium was supplemented with 0.54 μCi/ml of ^{3}H-BP[11] (sp act = 8.0

[7] From E. Leitz, Incorporated, New York, New York.

[8] From Keithly Instruments, Incorporated, Cleveland, Ohio.

[9] Kindly given to us by Dr. Hans Falk, National Institute of Environmental Health.

[10] From Falcon Plastics, Oxnard, California.

[11] From Amersham Searle, Des Plaines, Illinois.

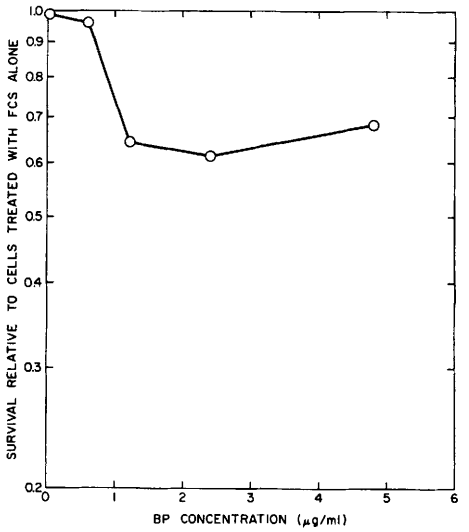

FIG. 2. Benzo(*a*)pyrene (BP) induced toxicity of V-79-4 hamster cells. The criterion of survival was colony forming ability.

Ci/mmole)]. In some cases cells were pretreated in the petri dishes with 0.6 μg/ml of BP for 12 hr and incubated for 1 hr without BP before being placed in the Columbia jars. After the desired times, coverslips were removed, washed five times in Hanks' balanced salt solution (HBSS) and fixed for 30 min in dilute (4%) formalin. For fluorescence analysis, coverslips were mounted in 60% glycerin–1 *N* NaOH solution. For liquid scintillation detection, they were dried at 85° for 30 min, added to scintillation vials containing 10 ml of Liquifluor scintillation fluid[12] and counted in a Beckman LS-200 liquid scintillation counter.

Measurement of BP toxicity. On the day prior to toxicity experiments, cultures were seeded at 1.7×10^5 cells/ml to disperse the cells. The next day cells were collected by trypsinization and diluted in two suspensions—150 and 300 cells/ml. The cells were added in 1.0-ml aliquots to 3.0 ml of preincubated media in 60-mm plastic petri dishes and allowed to attach by reincubating for 2 hr at 37° and 10% CO_2. BP-FCS was then added with a Biopet[13] to designated dishes which were then reincubated for 10–13 days at 37° and 5% CO_2. BP-media was allowed to remain in contact with cells for the duration of the experiment. In some experiments, BP dissolved in DMF was added directly (23) to petri dishes with no FCS intermediate. After the incubation period, colonies were washed twice in HBSS, fixed 10 min in absolute methanol, stained 30 min in dilute (1:15) Giemsa, and counted.

Results. BP toxicity. Figure 2 shows the toxic response of V-79-4 cells to increasing concentrations of BP. At low concentrations (0.6 μg/ml) there is little if any BP-induced toxicity. At concentrations between 1.2 and 4.8 μg/ml, there is a small (about 40%) but constant BP-induced lethality which continues to concentrations above the limit of BP solubility (Fig. 3). This leveling-off of toxicity suggested that there may also be a leveling-off of BP uptake.

Cellular BP distribution. After 30 min of BP treatment, the blue-violet fluorescence of BP was quite apparent in small perinuclear vesicles but not in the nucleus. Longer treatment times did not change this localization pattern except that these vesicles which appeared to be qualitatively similar to lysosomes had coalesced into larger vesicles after 1 to 3 days.

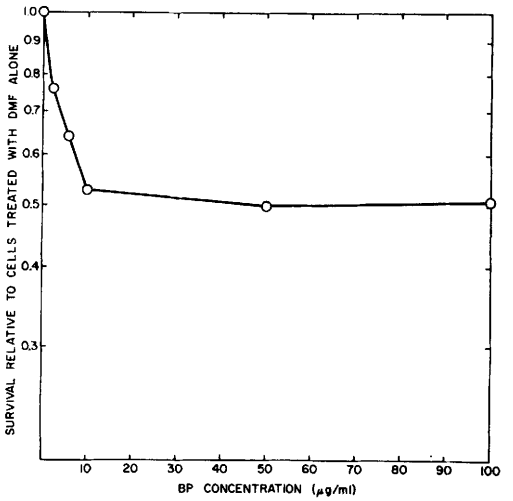

FIG. 3. Benzo(*a*)pyrene (BP) induced toxicity of V-79-4 hamster cells. The criterion of survival was colony forming ability. BP in *N,N*-dimethylformamide (DMF) was added directly to culture dishes to obtain higher BP concentrations.

[12] From New England Nuclear, Boston, Massachusetts.

[13] From Schwarz Bioresearch, Orangeburg, New York.

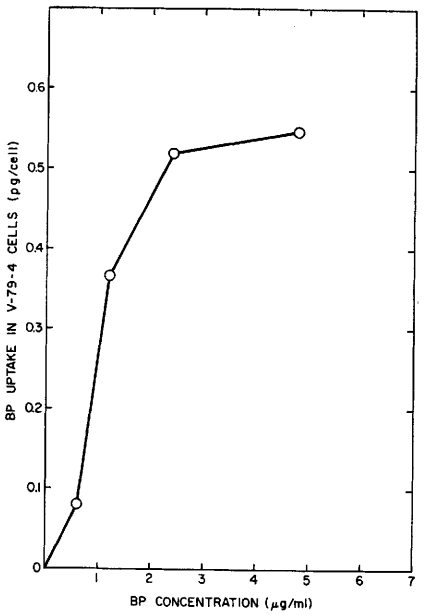

FIG. 4. Intracellular benzo(*a*)pyrene (BP) after 24 hr of treatment with various concentrations of BP.

BP uptake. At 24 hr, there is a leveling off of BP-uptake (Fig. 4) at BP concentrations above 1.2 μg/ml. Table I shows the time course of BP uptake into V-79-4 cells. There are three interesting points in Table I: (a) there is a dose-related amount of intracellular BP after any given BP treatment period; (b) at nonlethal concentrations, there is a definite maximum of 0.25 pg/cell at 1.0 hr in the amount of intracellular BP; and (c) at nonlethal concentrations, there is a significant ($p<0.1$) decrease in amount of BP per cell between 1.0 and 11 hr.

Analysis of BP uptake response at nonlethal concentrations. The time course of initial increase and subsequent loss of the characteristic BP fluorescence from cells could result from initial uptake followed by either true outward transport of BP or enzymatic induction and metabolism of BP to nonfluorescent entities. Binding of a significant fraction of cellular BP or metabolic products of BP to macromolecules in ways which quench fluorescence would be qualitatively similar to this latter possibility. These alternatives were checked by: (a) microfluorometric assay for possible metabolic formation of 3-OH BP; (b) investigating the BP uptake in BP-*pretreated* (*i.e.*, preinduced) cells; and (c) comparing the time course of uptake of tritiated BP to that of BP uptake measured microfluorometrically. Assay of green fluorescence (a measure of 3-OH BP production) in BP-treated cells showed no significant increase in fluorescence with time of BP treatment. Table II compares 3-OH BP production of V-79-4 cells with that of primary Syrian golden hamster fetus cells (38). The small amount of green fluorescence observed in V-79-4 cells can be accounted for as the tiny amount of long wavelength emission

TABLE I. BP Uptake[a] in V-79-4 Cells.[b]

BP conc (μg/ml)	Period of treatment (hr)					
	0.5	1.0	3.0	5.0	11.0	24.0
0.6	0.14	0.25[c]	0.10	0.091	0.065[c]	0.079
1.2	0.26	0.26	0.28	0.39	0.36	0.36
2.4	0.40	0.41	0.37	0.47	0.43	0.52
4.8	0.49	0.46	0.46	0.45	0.54	0.55

[a] In pg/cell.

[b] Average value for 4 expts.; approximately 60 cells measured/expt.

[c] This difference is statistically significant at $p < 0.10$.

TABLE II. Comparison of 3-Hydroxy BP Production[a] in V-79-4 Cells and Primary Hamster Cells[b] in Culture After Treatment with 0.6 μg/ml of BP.

Cell type	BP treatment period + period in BP free medium (hr)				
	1 + 1	5 + 1	11 + 1	24 + 1	48 + 1
Primary Syrian hamster	2.4	9.8	13.	48.	43.
V-79-4	1.5	1.6	3.3	2.5	3.6

[a] Values given in terms of nA of photomultiplier current per cell. Each value is the average of 3 expts.; 10 cells measured/expt.

[b] Data taken from Kouri *et al.* (38).

TABLE III. Average BP Uptake[a] by Normal Cells Which Were Pretreated for 12 Hours with 0.6 μg/ml of BP Compared with Uptake by Nonpretreated Cells.[b]

Expt.	BP conc (μg/ml)	Period of treatment (hr) 0.5	1.0	3.0	5.0	11.0	24.0
Pretreated	0.6	0.26	0.21	0.22	0.19	0.21	0.23
Nonpretreated	0.6	0.14	0.25	0.10	0.091	0.065	0.079

[a] In pg/cell.

[b] Average of 2 expts., each in duplicate; approximately 60 cells measured/duplicate.

from BP. If some other inducible enzyme were causing this loss of BP, then pretreatment with BP might induce this enzyme and cause a more rapid decrease of BP per cell. Table III shows BP uptake in cells pretreated for 12 hr with 0.6 μg/ml of BP. No rapid decrease in amount of BP per cell is evident. In fact, pretreatment interfered with extrusion of this carcinogen from cells. Table IV compares uptake of BP as measured by fluorescence with that measured by liquid scintillation detection of tritiated BP. Differences in results obtained by the two methods may result from heterogeneity in the population of cells and cellular debris assayed by liquid scintillation as contrasted with the healthy, attached cells selected for microfluorometry. Considering the diverse means of quantitiation, there is good agreement in amounts of BP uptake per cell when measured by those two techniques. In particular, both techniques indicate a drop in the amount of BP per cell during the period from 1.0 to 24 hr in BP media.

Discussion. The present results indicate that cellular uptake of BP can be reproducibly quantitated in absolute units using our microfluorometric technique. Use of standard fluorescent solutions in a hemocytometer for calibration makes this microfluorometric technique suitable for absolute assay of a wide range of fluorescent materials in cells. The sensitivity limit of this instrument is determined by the nonspecific background fluorescence which in V-79-4 cells corresponds to approximately 10^{-14} g of BP/cell. Previous qualitative observations by fluorescence microscopy (19, 22–27) have shown that polycyclic aromatic hydrocarbons are quickly taken up into small masses (phagolysosomes) in the cytoplasm of cells and not in the nucleus (28). Our observations confirm these earlier reports in the case of BP uptake and also agree very well with the average amounts of BP uptake/cell obtained by Morimura *et al.* (29), who used a rather elaborate extraction technique to quantitate BP in human FL amnion cells.

The results in Table I and Fig. 2 show that cells treated with 1.2 to 4.8 μg of BP/ml take up approximately similar amounts of BP and demonstrate similar degrees of lethality. However, treatment with 0.6 μg of BP/ml results in relatively small amounts of BP uptake and almost no lethality. Thus there appears to be a correlation between BP uptake and BP induced toxicity. It has been suggested by Allison and coworkers (19, 30,

TABLE IV. Comparison of BP Uptake[a] by V-79-4 Cells as Measured by Fluorescence and ^{3}H-BP Scintillation Detection.[b]

BP conc (μg/ml)	Period of treatment (hr) 0.5	1.0	3.0	5.0	11.0	24.0	48.0
Scintillation: 0.6	0.31	0.40	0.34	0.30	0.27	0.25	0.17
Fluorescence: 0.6	0.14	0.25	0.10	0.091	0.065	0.079	—

[a] In pg/cell.

[b] Values given as the average of 2 expts., each run in triplicate.

31) that the primary mode of action of BP toxicity involves the lysosomes unless the microsomal AHH is present. BP or molecules similar to BP have been reported to: (a) induce formation of new lysosomes *in vitro* (32), (b) accumulate within lysosomes if no corresponding lytic enzymes are present to digest it (33), (c) bind to particular glycolipids of the lysosomal membrane (34), and (d) decrease the stability of lysosomal membranes (19, 30, 31). The fluorescence measurements of V-79-4 cells at 522 nm (Table II) indicate that they produce no 3-OH BP and therefore lack AHH. Since these cells do accumulate significant amounts of BP in lysosomes, the observed toxicity in these cells would seem to be mediated by lysosomes. To further test this tentative conclusion, we attempted to determine whether BP could induce formation of new lysosomes by observing the number of lysosomes per cell after various BP treatment times. However, the heterogeneity in numbers and sizes of lysosomes prevented any significant results.

Decrease in cellular BP observed at 0.6 μg of BP/ml may be due to a process of lysosome extrusion reported by Woodin and Wienecke (35, 36). They demonstrated that leukocytes *in vitro* may extrude lysosomes into the medium by means of membrane fusion. More recently, Gross *et al.* (37), reported that hormones may be actively pushed out of cells under normal physiologic conditions. If this normal lysosomal extrusion process is involved in the loss of BP from V-79-4 cells, it appears to be inhibited at higher BP concentrations (1.2 to 4.8 μg/ml) which we also found to be toxic by survival criteria. This correlation therefore lends additional support to the idea of lysosomal mediation of toxicity.

Summary. This report states that using a new microfluorometric technique we can: (i) quantitate the amount of BP taken up by cultured mammalian cells, (ii) follow the time course of uptake of BP into these cells, (iii) correlate the amount of BP uptake with BP-induced cytotoxicity, (iv) observe BP extrusion by cells at low concentrations of BP, and (v) correlate this BP extrusion with reduced BP toxicity in these cells.

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