Biochemical Pharmacology, Vol. 28, pp. 651–655

0006-2952/79/0301-0651 $02.00/0

EFFECT OF METHYLMERCURY(II) ON THE SYNTHESIS OF DEOXYRIBONUCLEIC ACID, RIBONUCLEIC ACID AND PROTEIN IN HeLa S3 CELLS

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(*Received* 7 *April* 1978; *accepted* 24 *July* 1978)

Abstract—Exposing HeLa S3 cells to increasing concentrations of methylmercury $CH_3Hg(II)$ for varying periods of time results in an abrupt cessation of intracellular events, such as DNA, RNA and protein syntheses, once the organomercurial concentration in the growth medium has exceeded a certain threshold, the value of which lies between 1 and 10 μM. The synthetic activities, expressed as the ability of the cells to incorporate ^3H-labeled thymidine, uridine and leucine into the acid-insoluble fraction of the cells, decrease exponentially with time at a given methylmercury concentration and, at a given time, they cease in an abrupt, highly cooperative manner when plotted against the logarithm of the $CH_3Hg(II)$ concentration, yielding sigmoidally shaped dose–response curves. The inhibition of intracellular macromolecular synthesis is reversible up to a certain $CH_3Hg(II)$ level. For instance, cells that had been kept for 6 hr at $CH_3Hg(II)$ concentrations ranging from 2.5 to 10 μM did recover, albeit with lag periods that increased in length with increasing organomercurial concentration, as evidenced by their ability to continue RNA synthesis once the intoxicating medium had been removed. However, cells that had been exposed to 20 μM $CH_3Hg(II)$ and above did not recover, at least not at incubation periods up to 91 hr (4.3 generation times), after termination of the 6 hr methylmercury treatment. In general, macromolecular synthesis decreases in the sequence DNA ~ RNA > protein, followed by cell death as soon as protein synthesis has become inhibited to a major degree. The acute effects of $CH_3Hg(II)$ on intracellular DNA, RNA and protein syntheses were marginally different when HeLa S3 cells were exposed to methylmercury in three modes: as monolayers, as suspensions prepared from freshly trypsinized monolayer cells, and as suspension-cultures, demonstrating that, for instance, trypsin treatment of the cells increases their sensitivity toward methylmercury intoxication only slightly.

We have shown recently [1] that cultured human cervix carcinoma cells Hela S3, exposed to sufficiently high concentrations of the environmental pollutant methylmercury(II),* display an interesting dose–response behavior as far as their resistance toward this organomercurial is concerned: below a certain threshold value, viz. at $CH_3Hg(II)$ concentrations of 1 μM and below, the cells remain alive and exhibit the viability of control cells as evidenced by the trypan blue exclusion test [2] but at $CH_3Hg(II)$ concentrations of 10 μM and above, rapid cell death occurs. The abruptness of the transition of the cells from the live (i.e. trypan blue impermeable) state to the dead (dye permeable) state, comprising only a few hundredths log-M units of added organomercurial, was taken by us as being indicative of major changes occurring in the cellular membrane as a result of methylmercury intoxication.

As a sequel to the above work, we report now on the effect of $CH_3Hg(II)$ on intracellular DNA, RNA and protein syntheses. We have studied the incorporation of [methyl-^3H]thymidine, [5-^3H]uridine and L[4,5-^3H]leucine into DNA, RNA and protein, respectively, as a function of increasing organomercurial concentration in the growth media. HeLa S3 cells were exposed to methylmercury in three different modes: as monolayers, as suspensions prepared from trypsinized monolayers, and as suspension-cultures. This was done to see whether the mode of culture maintenance has any effect on methylmercury toxicity.

* Methylmercury(II), or simply methylmercury, denotes the chemical entity $CH_3Hg(II)$ without reference to any particular anion that might accompany this monofunctional (but divalent) mercuric derivative.

MATERIALS AND METHODS

Stock cell cultures

HeLa S3 cells were obtained as monolayer cultures from the Cell Culture Laboratory of the School of Public Health, University of California, Berkeley, CA. The cells were maintained both as monolayers (75 cm^2 plastic Falcon flasks—Becton, Dickinson & Co., Oxnard, CA) and as suspensions (500 ml Wheaton impeller flasks—Wheaton Scientific, Millville, NJ), and they were routinely passaged in Eagle's minimal essential medium (MEM-Joklik modified—Grand Island Biological Co. (GIBCO), Grand Island, NY). The medium was supplemented with 10% fetal calf serum (GIBCO) but, in contrast to previous practice [1], antibiotics were not added. Cell growth took place in water-saturated 95% air–5% CO_2 at 37°. A maximum cell density of 40×10^6 cells/flask could be obtained with monolayers; the cell density in the suspensions was maintained between 0.3 and 1.2×10^6 cells/ml. Cell viability was found to be better than 98 per cent, as judged from the trypan blue exclusion test. All cells were screened for mycoplasmal infection [3] utilizing the mycoplasm screening service of Irvine Scientific, Irvine, CA. Cells found to contain mycoplasmal contamination were not used.

Chemicals and other materials

Methylmercuric hydroxide, CH_3HgOH (97+%), was purchased from Alfa Products (Ventron Corp., San Leandro, CA). Its handling has been described elsewhere [1]. L[4,5-^{3}H]leucine (58 Ci/m-mole), [methyl-^{3}H]thymidine (47 Ci/m-mole), and [5-^{3}H]uridine (27 Ci/m-mole) were purchased from Amersham/Searle, Arlington Heights, IL. Trypsin (0.25%) was a product of GIBCO, while reagents such as phosphate-buffered saline (PBS) (all in mg/l: 8000 NaCl; 200 KCl; 200 KH_2PO_4; and 2160 Na_2HPO_4) (Mallinckrodt, St. Louis, MO) or toluene–PPO–POPOP–liquid scintillation fluor [4 g of 2,5-diphenyloxazole (PPO) and 0.05 g *p*-bis-[2-(5-phenyloxazolyl)]benzene (POPOP) dissolved in 1 liter of toluene] (Fisher Scientific Co., Pittsburgh, PA) were prepared from reagent grade chemicals. All other chemicals were of reagent grade also. The filters used in the labeling studies were Millipore HAWP filters of 0.45 μm pore size (Millipore Corp., Bedford, MA).

Methylmercury intoxication and precursor labeling studies

Monolayers. Cells were removed from one of the monolayer stock cell cultures by trypsinization and suspended in new medium. Then, 2 ml portions, each containing 0.15×10^6 cells/ml, were used to inoculate thirty Petri dishes (35 mm) each. The new cultures were permitted to become established by keeping them for 14–16 hr in the CO_2 incubator. One hr before commencement of methylmercury intoxication, the spent medium and unattached cells were removed by aspiration, 1 ml each of new medium added, and the incubation continued. Ten μl portions each of appropriate methylmercury stock solutions were then added to the cultures so that six sets, each composed of four dishes with the final methylmercury concentrations of 2, 4, 6 and 8 μM, respectively, were obtained. * A fifth dish (control) for each set was prepared by adding 10 μl water to the medium. Incubation was continued by keeping the sets for 1, 3, 6, 12, 24 and 36 hr, respectively, at 37° in water-saturated 95% air–5% CO_2. At the end of a given time period, 50 μl of a given isotope, dissolved in water at levels of 2.5 μCi [^{3}H]thymidine, 5 μCi [^{3}H]uridine, or 25 μCi [^{3}H]leucine, was added in about 30-sec intervals to each dish of a given set and incubation and labeling were stopped 30 min later by first aspirating the media, rinsing the cells gently with 1 ml each of ice-cold PBS, and, finally, by adding 2 ml each of ice-cold 5% (w/v) trichloroacetic acid (5% TCA) and keeping the Petri dishes on ice slush. Using a rubber policeman and a 1 ml Eppendorf pipet, the cells were collected on Millipore filters and, after rinsing dishes, pipet, and filters three times with 2 ml portions each of ice-cold 5% TCA, the filters were dried for about 30 min on a foil-covered hot plate and prepared for counting in a Beckman model 250 liquid scintillation counter. Higher levels of labeled leucine were employed since the medium is not free of this amino acid.

Suspensions prepared from trypsinized monolayer cultures. This refers to cell preparations in which the cells first are removed from a stock monolayer culture by trypsinization and, after being suspended in new medium and having methylmercury added immediately at the appropriate concentrations to the medium, are then kept in suspension by continuous agitation at 37° [1].

To 7 ml portions of such a suspension preparation, each portion with 0.3×10^6 cells/ml, 70 μl aliquots of stock methylmercury, made up with PBS, were added so that three sets of seven assay tubes each, one set per given isotope, were obtained with methylmercury concentrations of 1, 2, 4, 6, 8, 10 μM, and 10 mM respectively. An eighth tube was always made up as a control by adding 70 μl PBS to a 7 ml suspension sample. Each set, after gassing with 5% CO_2, was incubated for the times listed above under "Monolayers". At the end of each given time period, a 1 ml aliquot was removed under sterile conditions from each tube for labeling purposes while the remainder of the suspensions, after renewed gassing, was subjected to continued incubation until the next time point was reached.

Labeling was performed as described above under "Monolayers", the only differences being that medium removal and washing involved vacuum filtration, making sure that only gentle suction was applied and that the isotopes were employed at slightly reduced levels, viz. 1 μCi [^{3}H]thymidine, 2 μCi [^{3}H]uridine, and 10 μCi [^{3}H]leucine.

Suspension-cultures. Cells were removed from an established and exponentially growing suspension-culture (generation time $T_g = 21$ hr) by centrifugation and incubated anew with fresh medium (5% CO_2 atmosphere) at a cell density of $(0.6–0.8) \times 10^6$ cells/ml. After 18 hr of incubation, the cells were counted, fresh medium was added so that the final cell density amounted to 0.3×10^6 cells/ml and, after gassing again with 5% CO_2, incubation was continued for another 20 min. The cells were then divided into 7 ml aliquots and treated with the organomercurial as described above under "Suspensions".

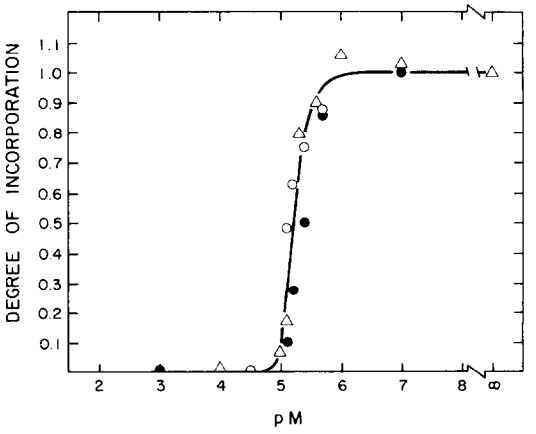

Fig. 1. Change in intracellular DNA synthesis as a function of $CH_3Hg(II)$ concentration after 12 hr of incubation. DNA synthesis is expressed as incorporation of [^{3}H]thymidine, in fraction of the control (pM ∞), into acid-insoluble cellular material. Key: pM $\equiv -\log [CH_3HgOH]_{added}$; (○) monolayer cells; (●) suspension cells; and (△) suspension-culture cells. For further details, see text.

* The dilution error of about 1 per cent was ignored here as in all other handling to be reported further below.

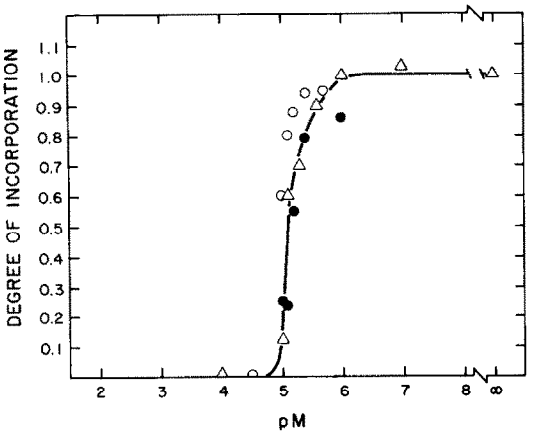

Fig. 2. Change in intracellular RNA synthesis as a function of $CH_3Hg(II)$ concentration after 12 hr of incubation. RNA synthesis is expressed as incorporation of [^{3}H]uridine, in fraction of the control (pM ∞), into acid-insoluble cellular material. Key: pM ≡ − log $[CH_3HgOH]_{added}$; (○) monolayer cells; (●) suspension cells; and (△) suspension-culture cells. For further details, see text.

Recovery experiments. Fifty-ml portions of cells grown in suspension-culture, each portion at a density of 0.3×10^6 cells/ml, were inoculated with 0.5-ml portions of stock $CH_3Hg(II)$ dissolved in PBS, at concentration levels of 2.5, 5.0, 7.5, 10, 20 and 30 μM, respectively, and kept for 6 hr at 37° (5% CO_2) in a shaker bath. The control was prepared by adding 0.5 ml PBS. At times corresponding to 3 and 5 hr of incubation in the presence of methylmercury, 1 ml aliquots were removed from each suspension and assayed in duplicate for their ability to incorporate [^{3}H]uridine into acid-insoluble material. This served as a control to see whether the decrease of RNA synthesis proceeded as expected. At 6 hr, the cells were removed from the intoxicating media by centrifugation, washed twice with fresh medium (void of the organomercurial), and resuspended in 46 ml each (the volume of the intoxicating medium at 6 hr) of new methylmercury-free medium. After gassing with CO_2, incubation was continued and each suspension was assayed for [^{3}H]uridine incorporation at 2, 5, 18, 24, 47, 67 and 91 hr after termination of the methylmercury treatment. The cells were kept in the log phase by renewing the medium and by splitting the cells at appropriate intervals.

RESULTS

Exposing HeLa S3 cells for 12 hr to increasing concentrations of $CH_3Hg(II)$ leads to cessation of intracellular DNA synthesis, as shown in Fig. 1. The incorporation of exogenous [^{3}H]thymidine, expressed as a fraction of the control (pM ∞), into acid-insoluble portions of the cells decreases abruptly between pM 6 and 5, where pM ≡ − log $[CH_3HgOH]_{added}$. The open-circled points refer to cells that had been maintained as monolayer cultures whereas the closed-circled and triangular points represent freshly trypsinized cells (suspensions) and cells that had been kept routinely in suspension (cf. Materials and Methods). It is of interest to note that minute changes in the organomercurial concentration in the medium, e.g. a 3- to 4-fold increase, completely inhibit DNA synthesis. Similar results are obtained with respect to intracellular RNA and protein syntheses: Fig. 2, for instance, shows the effect of methylmercury on the uptake of [^{3}H]uridine after 12 hr of incubation.

The time course of the methylmercury-induced inhibition of DNA synthesis at a given $CH_3Hg(II)$ concentration, viz. at 8 μM (pM 5.1), is shown in Fig. 3. The incorporation of [^{3}H]thymidine into acid-insoluble material decreases exponentially with time. Least sensitive in this respect, particularly during the early stages of incubation, were cells that had been kept as monolayer cultures while cells that were in suspension-culture during methylmercury treatment display a higher degree of sensitivity. This finding might simply be the result of the slow diffusion of the $CH_3Hg(II)$ through the monolayer culture medium since after 24 hr of incubation there is little difference among the three culture types as far as their susceptibility to methylmercury is concerned. It is of interest to note that trypsinized cells (suspensions) are only marginally more sensitive than are true suspension-culture cells.

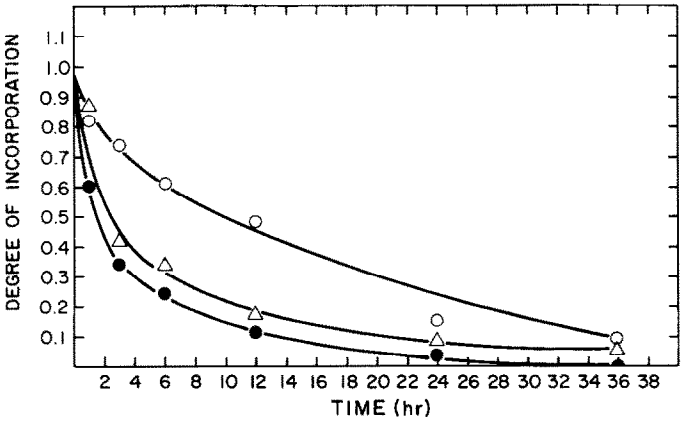

Fig. 3. Time course of DNA inhibition at a given $CH_3Hg(II)$ concentration in the medium. $[CH_3HgOH]_{added}$: 8 μM (pM 5.1). Key: (○) monolayer cells; (●) suspension cells; and (△) suspension-culture cells. DNA synthesis is expressed as [^{3}H]thymidine incorporation (in fraction of the control, pM ∞).

The decay curves shown in Fig. 3 for the incorporation of [^{3}H]thymidine by HeLa S3 hold also for the incorporation of [^{3}H]uridine or [^{3}H]leucine. However, at a given methylmercury concentration, macromolecular synthesis decreases in the order DNA ~ RNA > protein, irrespective of the mode of culture maintenance. For instance, in Fig. 3, cells grown in suspension-culture (triangles) display, after 3 hr of incubation, a residual DNA synthesis that amounts to about 40 per cent of the control. Under the same conditions, RNA and protein syntheses (not shown) proceed with about 50 and 90 per cent efficiency of the control, respectively, while after 36 hr of incubation the relation DNA:RNA:protein = 5:10:20 (in per cent of control) holds.

Figure 4 correlates some of the results gathered in this work with cell viability results obtained previously [1]. Suspension cells were exposed for 6 hr to varying concentrations of $CH_3Hg(II)$ and their ability to incorporate the three labeled precursors into acid-insoluble material was determined. As can be seen, there is an abrupt decline of DNA and RNA syntheses between pM 6 and 5 followed by a cessation of protein synthesis between pM 5 and 4.5. As is seen further, suspension cells, cultured and exposed to methylmercury under nearly identical conditions [1], become permeable to trypan blue right after protein synthesis has become inhibited to a large degree.

In an attempt to identify more closely the organomercurial concentration in the medium above which HeLa S3 cells do not recover from injury once they have been removed from the $CH_3Hg(II)$, the cells were first incubated for 6 hr in $CH_3Hg(II)$ concentrations ranging from 2.5 to 30 μM and then in methylmercury-free medium for periods up to 91 hr (4.3 generation times) while, at the same time, they were scanned periodically for their ability to incorporate [^{3}H]uridine. As is seen in Fig. 5, control cells and cells that had been exposed to 2.5 and 5.0 μM methylmercury (pM 5.6 and 5.3) continued RNA synthesis at comparable rates, i.e. did not display any permanent inhibition. Cells that had been kept in the presence of 7.5 μM methylmercury (pM 5.1) needed more time to resume RNA synthesis while cells maintained at pM 5.0 remained stationary for about 48 hr before synthesis commenced. Cells kept at 20 and 30 μM $CH_3Hg(II)$ (pM 4.7 and 4.5) did not recover: [^{3}H]uridine counts started out at about 300 cpm and remained at this level for about 18 hr after $CH_3Hg(II)$ removal but became indistinguishable from background noise thereafter (not shown in the figure due to the large difference in scale).

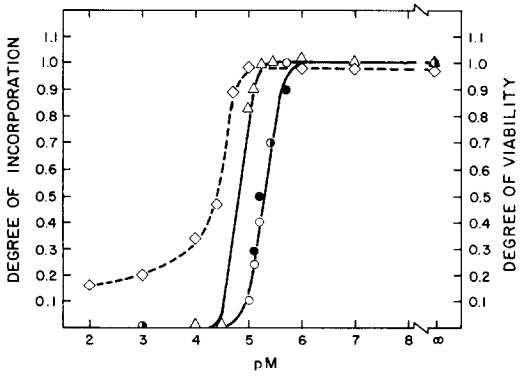

Fig. 4. Comparison of methylmercury-induced inhibition of DNA (○), RNA (●) and protein (△) syntheses [left-hand ordinate, expressed in fraction of the control (pM ∞)] with change in cell viability (◌) (right-hand ordinate, expressed as fraction of the number of live cells found in the sample as evaluated via the trypan blue exclusion test) as a function of methylmercury concentration. The half-closed symbols (◑) represent overlap of open and closed-circled data points. The DNA, RNA and protein data pertain to 6 hr of incubation in the presence of $CH_3Hg(II)$. The viability data were taken from Ref. 1; they were gathered after 5 hr of incubation in the presence of $CH_3Hg(II)$. pM $\equiv -\log [CH_3HgOH]_{added}$. All data were obtained with suspension cells; macromolecular synthesis is again expressed in terms of the incorporation of the ^{3}H-labeled appropriate precursors by the cells. For further details, see text.

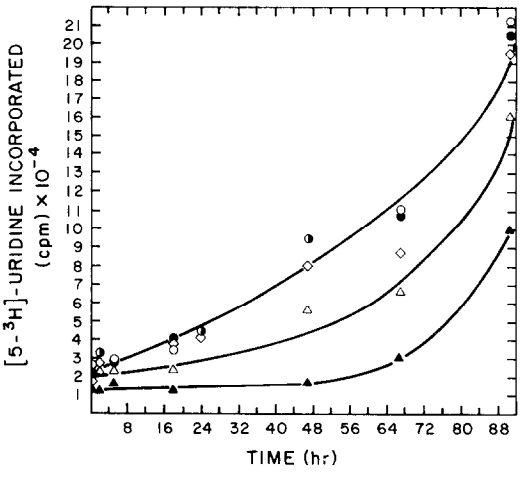

Fig. 5. Recovery of HeLa S3 suspension-culture cells from methylmercury intoxication. The cells were first treated for 6 hr with the indicated methylmercury concentrations and then reincubated with fresh methylmercury-free medium (time 0 hr) for the indicated periods of time. Cell recovery is expressed in terms of renewed RNA synthesis ([^{3}H]-uridine incorporation). Left-hand ordinate: counts/min. Key: (○) control (pM ∞); (●) 2.5 μM (pM 5.6); ( ) 5.0 μM (pM 5.3); (△) 7.5 μM (pM 5.1); (▲) 10 μM (pM 5.0); and (◑) overlap of open and closed-circled data points. Not shown are the 20 μM (pM 4.7) and 30 μM (pM 4.5) data since their initial counts are below 500 cpm and become indistinguishable from background noise thereafter. For further details, see text.

DISCUSSION

Our previous finding [1] that HeLa S3 cells exposed to sufficiently high concentrations of methylmercury die abruptly, within an extremely narrow concentration range of the organomercurial in the medium, is corroborated by the results of this study. The acute effects of $CH_3Hg(II)$ on intracellular metabolic events such as DNA, RNA and protein syntheses are similar to the effects on cell viability: below a certain threshold value, e.g. at $CH_3Hg(II)$ concentrations of 1 μM and below, the cells remain alive and display the viability and metabolic activity of control cells, but at $CH_3Hg(II)$ concentrations of 10 μM and above, rapid cessation of macromolecular synthesis in the sequence DNA ~ RNA > protein occurs, followed, at still

higher concentrations, by uptake of trypan blue into the cell interior (cf. Fig. 4). In fact, at $CH_3Hg(II)$ concentrations of 20 μM and above, irreversible cell damage seems to have taken place as shown by the inability of the cells to proceed with RNA synthesis once the intoxicating medium has been removed (cf. Fig. 5).

There is no doubt that both DNA and RNA syntheses are considerably more susceptible to inhibition by methylmercury than is protein synthesis (Fig. 4). However, whether DNA synthesis is more sensitive than RNA synthesis, or vice versa, is less clear-cut. While we have found many instances that indicate DNA synthesis is the more sensitive macromolecular event, which suggests, therefore, that DNA synthesis is the intracellular activity which is inhibited first, we have also obtained results that show RNA synthesis is at least as sensitive as DNA synthesis to methylmercury.

To our knowledge, there is at present not much information available from the literature that permits comparison of the results obtained in this study with results obtained by others. The one publication we feel comes closest to the gist of our own investigation is the study by Nakazawa *et al.* [4]. They studied the effects of $CH_3Hg(II)$ on survival and intracellular DNA and RNA syntheses of mouse leukemic L5178Y cells. Apart from differences in culturing conditions, the major difference is that they exposed their cells only for 1 hr to varied concentrations of methylmercury and that labeling and intoxication were performed simultaneously. By and large, their results are in agreement with the results obtained by us; this is particularly true as far as the viability (in their case plating efficiency, in our case trypan blue staining) of the two cell types is concerned. While L5178Y cells appear to be slightly more sensitive to methylmercury than HeLa S3 cells are (by a concentration factor of about three), their survival data, when plotted in the fashion employed by us, also yield a highly cooperative dose–response curve. However, our results differ considerably from theirs concerning the incorporation of labeled precursor. In their case, DNA and RNA syntheses decrease gradually when plotted against pM, in fact, covering about 2 pM units when going from 100 per cent activity to complete inhibition (their Figs. 4 and 6), while in our case, complete inhibition is accomplished within 1 μM unit (cf. Figs. 1 and 2 this work). Thus, whereas our labeling data parallel the viability data (cf. Fig. 4 this work), their incorporation data are at variance with their survival data in which, for instance, the decrease from 80 to 20 per cent survival proceeds within about 0.5 pM unit (their Fig. 2). We have no explanation to offer for this different behavior other than to suggest that their intoxication period was too brief to show cooperative effects as far as the breakdown of the intracellular synthetic events is concerned.

The $CH_3Hg(II)$ concentrations listed here represent total mercury in the medium and not levels found per cell. Consequently, we are presently unable to correlate the dose–response data, e.g., the pM value pertaining to the 50 per cent viability point or the pM values indicating 50 per cent inhibition of DNA, RNA and protein syntheses, with toxic *in vivo* doses known from LD_{50} data involving whole animals. Current research is in progress to measure the internal methylmercury concentration and to identify its subcellular localization.

Acknowledgement—The HeLa S3 cells used in this work were provided by Contract E-73-2001-NO1 within the Special Virus-Cancer Program, NIH, PHS, through the courtesy of Mr. J. Weaver of the Cell Culture Laboratory of the School of Public Health, University of California, Berkeley. This work was supported by U.S. Public Health Service Grant 1 R01 ES01115.

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