# EFFECTS OF INSECTICIDES ON MAMMALIAN CELLS AND VIRUS INFECTIONS

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Evaluation of the safety of a chemical in our environment, particularly a biologically active substance such as a pesticide, requires ideally a complete knowledge of its metabolism and of the physiological effects it produces, especially at the low exposure levels generally encountered. Such knowledge demands techniques that will reveal low level and subtle effects.

The ability to maintain *in vitro* cultures of a variety of tissue cells derived from many species of animals makes possible the study of biological effects in relatively simple systems. Such studies may provide leads for promising investigations in intact animals; conversely, indications from animal studies may be followed up intensively with the cell cultures that are free of the sometimes obscuring factors from the activities of the circulatory, endocrine, and nervous systems. Clearly, the cell culture system cannot replace the whole animal, but it does offer possibilities of yielding additional insights useful in the evaluation of potential hazard. One such possibility is that comparative cytotoxicity in cell cultures from various species, including man, would correlate sufficiently with comparative toxicity in the intact animal of different species to help select the experimental animal with tissue susceptibility most like that of man. Such knowledge, together with information on the comparative metabolism of the substance in various species and man, might be useful in extrapolating findings from experimental animals to the human situation with greater confidence than is now possible.

The persistence in the body of such compounds as DDT, dieldrin, lindane, endrin, and heptachlor[1-5] and the consequent continuous exposure of tissue cells to these substances and their metabolites indicated the need for more information regarding the response of cells to insecticides during acute and chronic exposure. Although more comparative data than now available are needed to establish the usefulness of such studies, we believe the results[6-11] of our exploratory efforts are of interest for the evaluation of environmental hazards associated with the use of insecticides.

## Acute Toxicity Studies

### *Cell Cultures*

Established cell strains of human and mouse origin were used—human cells: the Chang liver strain derived from normal liver tissue and the HeLa strain derived from a cervical carcinoma; mouse cell cultures: the mouse liver (NCTC #1469) strain and the mouse skin fibroblasts (L-929) strain. Cultures were maintained in Eagle's basal medium[12] or Medium-199[13] supplemented with 10 per cent serum. In all tests the medium contained 100 units/ml penicillin and 100$\mu$g/ml streptomycin. For cytotoxicity tests, cells were suspended in growth medium at a concentration of $8.5 \times 10^4$ cells per ml, and 1 ml volumes were planted in culture tubes. Cultures were incubated in a stationary position at 37°C for 2 or 3 days until a confluent cell monolayer developed.

### *Insecticidal Compounds and Test Procedures*

All commercial insecticides used in this study were purified compounds. Their chemical names and purity are indicated in TABLE 1.

For testing, the water-soluble compounds, Dipterex® and Cygon®, were dissolved in the medium directly. All other compounds were water-insoluble and were first dissolved in ethyl alcohol and then diluted 100-fold in the medium. As determined in previous experiments, an ethyl alcohol concentration in the medium below 0.1 per cent was not toxic to the cells. Insecticide stock solutions were adjusted to pH 7.5 with $NaHCO_3$ solutions, when necessary.

### *Test Procedures*

The insecticide-treated cultures were incubated for 24 and 48 hr on a roller drum or in a stationary position at 37°C. Microscopic examination of the cells

TABLE 1

INSECTICIDAL COMPOUNDS

| Trade Name | Chemical Name | Purity (%) |
|---|---|---|
| | *Organophosphorus Compounds* | |
| Cygon* | O,O-dimethyl S-(N-methylcarbamoylemethyl)-phosphorodithioate | 99.8 |
| Dipterex®** | O,O-dimethyl (1-hydroxy-2,2,2-trichloroethyl)-phosphonate | 100 |
| DI-Syston®** | O,O-diethyl S-(2-ethylthio)ethyl phosphorodithioate | 96.9 |
| Malathion* | O,O-dimethyl S-(1,2-dicarbethoxyethyl)phosphorodithioate | 99.6 |
| | *Organochlorine Compounds* | |
| Bulan*** | 2-nitro-1,1-bis(p-chlorophenyl)butane | 98 |
| Chlordane† | 1,2,4,5,6,7,8,8-octachloro-2,3,3a,4,7,7a-hexahydro-4,7-methanoindene | Reference grade |
| o,p,DDD†† | 2-O-chlorophenyl-2-p-chlorophenyl-1,1-dichloroethane | Technical grade |
| p,p'DDD†† | 2,2-bis(p-chlorophenyl)-1,1-dichloroethane | Technical grade |
| DDT†† | 1,1,1-trichloro-2,2-bis(p-chlorophenyl)-ethane | Technical p,p'isomer 77.2 |
| Heptachlor† | 1,4,5,6,7,8,8-Heptachloro-3a,4,7,7a-tetrahydro-4,7-endomethanoindene | 97.8 |
| Kelthane®††† | 4,4'-dichloro-α-(trichloromethyl)benzhydrol | 96 |
| Methoxychlor§ | 1,1,1-Trichloro-2,2-bis(p-methoxyphenyl)ethane | Analytical standard 100 |
| Ovex§§ | p-chlorophenyl p-chlorobenzenesulphonate | 95.14 |
| Perthane®††† | 1,1-Dichloro-2,2-bis(p-ethylphenyl)ethane | 100 |
| Prolan*** | 2-Nitro-1,1-bis(p-chlorophenyl)propane | 98 |
| | *Dinitrophenol Compounds* | |
| Dinitro-o-cyclohexylphenol§§ | 2-cyclohexyl-4,6-dinitrophenol | 98 |
| Karathane®††† | 2-(1-methylheptyl)-4,6-dinitrophenylcrotonate | 88.4 |

* American Cyanamid Co., Princeton, N. J.
** Chemagro Corp., Kansas City, Mo.
*** Commercial Solvent Corp., New York, N. Y.
† Velsicol Corp., Chicago, Ill.
†† Edcan Labs., South Norwalk, Conn.
††† Rohm and Haas Co., Philadelphia, Penn.
§ E. I. DuPont Co., Wilmington, Del.
§§ Dow Chemical Co., Midland, Mich.

revealed the cytopathogenic effect which, in general, was characterized by progressive morphologic changes leading to the destruction of the cells. Destruction or morphologic alteration of 75 to 100 per cent of the cells was classified as a 4+ reaction; of 50 to 75 per cent as 3+; and of 25 to 50 per cent as 2+. The lowest concentration of an insecticide that affected 50 per cent of the cells during the incubation period of 48 hr was expressed as the toxic dose 50 per cent ($TD_{50}$).

The growth of the culture was measured by determination of total cell protein.[14] Concentrations of an insecticide that inhibited the growth by 10 and 50 per cent during the incubation period of 48 hr were designated as the inhibitory doses 10 per cent and 50 per cent ($ID_{10}$ and $ID_{50}$) and calculated by the method of Litchfield and Wilcoxon.[15]

*Comparative Cytoxicity.* The results of acute toxicity based on the cytopathogenicity and growth inhibition (FIGURE 1) showed a close correlation. For example, the growth curves of the human liver cell cultures treated with Dipterex® at 100 μg/ml indicated a complete growth inhibition and a 4+ cytopathogenicity in the 24-hr tests. At 48 hr the cultures were almost completely destroyed. In the second culture group exposed to 50 μg/ml, the growth was inhibited 35 per cent at 24 hr and 50 per cent at 48 hr. The corresponding degrees of cytopathogenicity were classified as 2+ and 3+ reactions.

TABLE 2 summarizes the cytotoxicity levels.[6] In Chang liver cells, Cygon was the least toxic compound, followed in order of decreasing toxicity by Dipterex, malathion, and DI-Syston®. The same sequence of toxicity was evident in HeLa cells. Comparatively, the toxicity of DI-Syston in human liver cells is about 34 times that of Cygon, 7 times that of Dipterex, and 3 times that of malathion.

The toxicity of malathion and several of its molecular fragments have been studied also by Wilson and Walker.[16] Since the growth of chick embryo cells was inhibited at 10 μg/ml of malathion, these cells appear to be in the same range of susceptibility as the human liver strain.

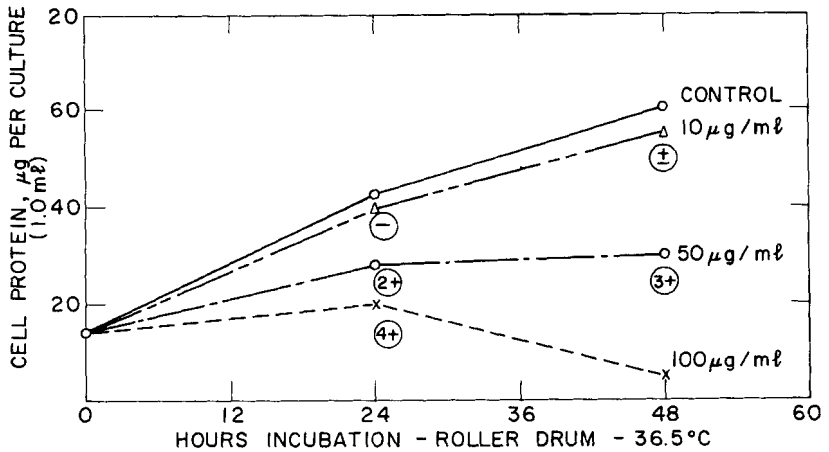


FIGURE 1. Effect of Dipterex on growth of human Chang liver cells during 24-hr and 48-hr incubation periods. Cytotoxicity is indicated by scores from − to 4+. Destruction or alteration of 75–100% of the cells is 4+; 50 to 75% is 3+; 25 to 50% is 2+; up to 25% is 1+; slight abnormality of questionable significance is ±; no change is −. (*From* Gabliks, J. & L. Friedman. 1965. Proc. Soc. Exp. Biol. Med. **120**: 163–168.)

TABLE 2

COMPARATIVE CYTOTOXICITY OF INSECTICIDES TO HUMAN CHANG LIVER AND HELA CELLS§

| Insecticide | Liver cells | | | HeLa cells | | |
|---|---|---|---|---|---|---|
| | Cytopathogenicity | Growth Inhibition | | Cytopathogenicity | Growth Inhibition | |
| | $TD_{50}$ | $ID_{50}$ | $ID_{10}$ | $TD_{50}$ | $ID_{50}$ | $ID_{10}$ |
| | μg/ml | | | μg/ml | | |
| | *Organophosphorus Compounds* | | | | | |
| Cygon | 100 | 170 | 50 | 500 | 220 | 110 |
| Dipterex | 50 | 35 | 10 | 70 | 22 | 10 |
| Malathion | 10 | 15 | 10 | 20 | 13 | 2 |
| DI-Syston | 1 | 5 | 1 | 15 | 9 | 7 |
| | *Organochlorine Compounds* | | | | | |
| Methoxychlor, Prolan, p,p'-DDD* o,p'-DDD,DDT(technical, Heptachlor, Perthane, Bulan† | | | | | | |
| Chlordane | 10 | 10 | 5 | 20 | 22 | 10 |
| Kelthane | 5 | 6 | .5 | 50 | 23 | 9 |
| Ovex | 5 | 5 | — | — | — | — |
| | *Dinitrophenol Compounds* | | | | | |
| Karathane | .5 | .3 | .1 | 3 | 4 | 1 |
| Dinitro-o-cyclohexylphenol | 1 | 1 | .1 | — | — | — |

* For these compounds, cytopathogenicity—$TD_{50}$ (μg/ml) = 1000, and were tested in suspension.

† For these compounds, cytopathogenicity—$TD_{50}$ (μg/ml) = 100, and were tested in suspension.

§ GABLIKS, J. & L. FRIEDMAN. 1965. Proc. Soc. Exp. Biol. Med. **120**: 163.

Cygon, Dipterex, malathion, and DI-Syston are known as potent inhibitors of acetylcholine esterase. Although most of the acute toxic effects of organophosphates in animals are probably due to inhibition of acetylcholine esterase in nervous tissue, other enzymes in other tissues are also affected by these agents.[17] The presented results suggest that the toxicity of organophosphorus compounds may be caused by their interference with the activities of esterases present in cultivated cells.

The organochlorine compounds, DDT, chlordane, Kelthane,® and Ovex®, were toxic at 70 μg, 10 μg, 6 μg, and 5 μg/ml, respectively. Generally, liver cells were more susceptible than HeLa cells.

The dinitrophenol compounds, Karathane® and dinitro-o-cyclohexylphenol, were the most toxic compounds used in this study. The $ID_{50}$ of Karathane was 0.3 μg/ml in Chang liver cells and 4 μg/ml in HeLa cells. In comparison to the least toxic organophosphorus compound, Karathane was 566 times as toxic as Cygon.

### *Malathion*

TABLE 3 shows comparative cytotoxicities of DI-Syston, Dipterex, Cygon, malathion, chlordane and DDT to mouse and human cell cultures.[10] Among the organophosphorus compounds tested, malathion, which was very toxic to human Chang liver cells, showed the lowest toxicity in mouse liver cells; it is also known as one of the least toxic organophosphorus compounds in the mouse.

TABLE 3

COMPARATIVE CYTOTOXICITY OF INSECTICIDES TO CELL CULTURES

| Insecticide | Growth Inhibitory Levels ($ID_{50}$ — μg/ml/culture) | | | $ID_{50}$ mg/kg Mouse (per OS)§ |
|---|---|---|---|---|
| | Mouse Liver 1469* | Mouse Skin L-929* | Human Liver Chang† | |
| | *Organophosphorus Compounds* | | | |
| DI-Syston® | 11 | 44 | 5 | 3–10 |
| Dipterex® | 2 | 56 | 35 | 500 |
| Cygon® | 310 | 370 | 170 | 60 |
| Malathion | 1804 | 106 | 15 | 720–3300 |
| | *Organochlorine Compounds* | | | |
| Chlordane | 1 | 53 | 10 | 430 |
| DDT | 125 | 128 | 70 | 150–400 |

* GABLIKS *et al.* 1967. Proc. Soc. Exp. Biol. Med. **125:** 1002–1005.
† GABLIKS, J. & L. FRIEDMAN. 1965. Proc. Soc. Exp. Biol. Med. **120:** 163.
§ NEGHERBON, W. O. 1959. Handbook of Toxicology. Vol. III. Philadelphia: W. B. Saunders Co.

In the organochlorine group, there was general agreement among the three cell lines. In all cases, chlordane was more toxic than DDT.

The observed differences in susceptibility of human and mouse liver cells to malathion and other organophosphorus compounds may be attributed partly to the rates of their inactivation to nontoxic derivatives and partly to their transformation to more potent derivatives.[18] Mouse liver cells were 11 times more susceptible to malaoxon than to malathion. The cytotoxicity results summarized in TABLE 4 suggest that the marked resistance of mouse liver cells to malathion is caused primarily by their inability to oxidize malathion to malaoxon, whereas Chang liver cells appear to be able to transform malathion to malaoxon and are accordingly highly susceptible.[10]

### *Dipterex*

The toxicity of an organophosphorous insecticide is also dependent on the rate of its detoxification, as shown with Dipterex. The data for Chang liver are illustrated in FIGURE 2. At 50μg/ml, the fresh Dipterex was found to be about 50 percent more toxic than the same concentration of Dipterex previously exposed to Chang liver cells. When the same preparations were injected into mice intravenously, only those mice that received solutions of the fresh Dipterex showed toxic effects. These results suggest inactivation of Dipterex by Chang liver cells. Dipterex, as reported by DuBois and Cotter, exerts an anticholinesterase action

TABLE 4

COMPARATIVE CYTOTOXICITY OF MALAOXON TO MOUSE AND HUMAN LIVER CELLS AT 24, 48, AND 72 HOURS OF INCUBATION*

| Cell Culture | Growth Inhibitory Levels $ID_{50}$ — μg/ml | | |
|---|---|---|---|
| | 24 hr | 48 hr | 72 hr |
| Mouse liver – NCTC 1469 | 200 | 160 | 5 |
| Human liver – Chang strain | 15 | 20 | 50 |

* GABLIKS *et al.* 1967. Proc. Soc. Exp. Biol. Med. **125:** 1002–1005.

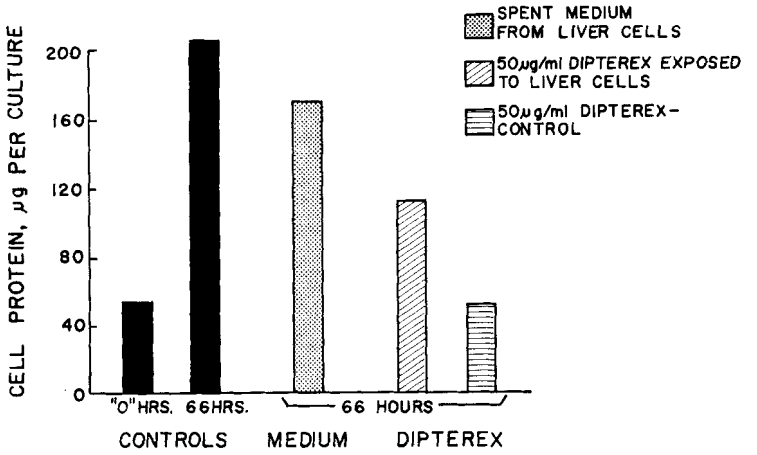


FIGURE 2. Detoxification of Dipterex by human liver Chang strain cells. Dipterex was exposed to Chang liver cells for 24 hr and its toxicity measured as the growth inhibitory effect on Chang liver cells during 66 hr of incubation. Growth in the presence of Dipterex and the control medium is expressed as increase of total cell protein per culture.

*in vitro* and *in vivo* and undergoes rapid detoxification in mammals, thus accounting for its low toxicity and short duration of action.[19]

### *Growth Stimulation*

In the acute toxicity experiments, some of the organophosphorous compounds stimulated growth of human and mouse liver cells when tested below the acute toxicity levels. The results obtained in mouse liver cells are summarized in FIGURE 3. Cygon at 50 $\mu$g/ml, level increased growth up to 190 percent; DI-Syston at 1 $\mu$g/ml, up to 130 percent; and malathion at 50 $\mu$g/ml, up to 160 percent of the control. Although not conclusive, preliminary experiments with Cygon in mice suggest that at low levels of intake there is a slight stimulation of growth.

## CHRONIC TOXICITY STUDIES

The chronic toxicity studies were conducted in human and mouse cells. Cells were suspended in the medium at $1 \times 10^5$ cells/ml, and 5 ml volumes were planted in Falcon 25 $cm^2$ plastic tissue culture flasks. Insecticides were incorporated in the medium at the time of planting. The insecticide-containing medium was changed every third day, or as required. When the cultures showed a full cell monolayer, cells were harvested by the trypsinization method and transplanted to new bottles in the same manner.

HeLa cells were exposed to Cygon, Dipterex, malathion, DI-Syston, chlordane, Kelthane, and Karathane for 107 days. The initial concentrations of insecticides were 1/20 to 1/200 of the $ID_{10}$ levels of the acute cytotoxicity. The appearance and degree of cytotoxicity were dependent on the concentration of the insecticide and the length of the exposure period.

The chronic cytotoxicity results during the exposures for the first 84 days are summarized in TABLE 5.[7] The first column indicates the nontoxic concentrations that did not induce any detectable signs of toxicity. The minimal toxic dose (MTD) levels at which insecticides induced cytopathic effects and growth inhi-

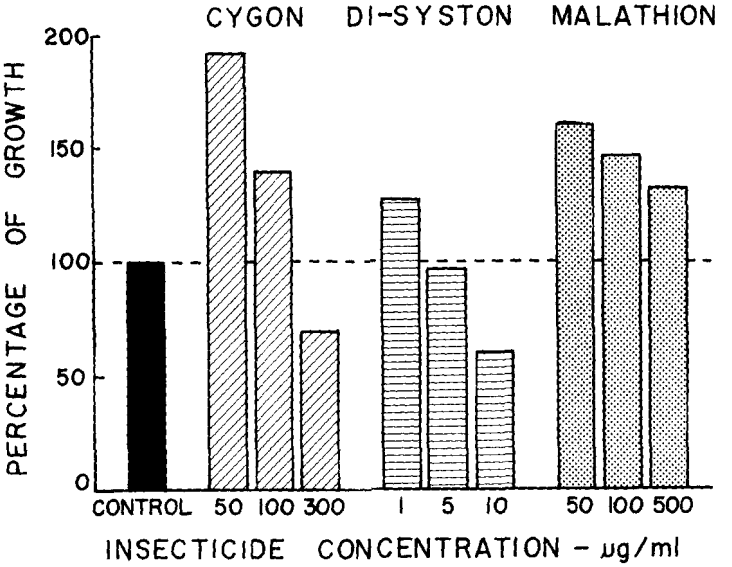


FIGURE 3. Growth stimulation of mouse liver NCTC 1469 strain cells by Cygon, DI-Syston, and malathion. The growth was measured as the increase of total cell protein per culture and expressed as percentages of the control values for means of comparison. (Gabliks *et al.* 1967. Proc. Soc. Exp. Biol. Med. **125**: 1002–1005.)

bition were: Cygon, 20 $\mu$g/ml; Dipterex, 10 $\mu$g; DI-Syston, 5 $\mu$g; Kelthane, 5$\mu$g; Karathane, 5 $\mu$g; malathion, 0.1$\mu$g; and chlordane, 0.1$\mu$g. If the magnitudes of the chronic and acute toxicities are compared, it is evident that most insecticides are significantly more toxic during chronic exposures. On continued incubation at the MTD levels or above, the HeLa cells become adapted to grow normally in the presence of high concentrations of insecticides, as shown by the tolerated toxic concentrations (TTC) listed in TABLE 5. At the TTC levels, the Dipterex, malathion, and chlordane cultures always showed an increased number of large, rounded, abnormal cells that contained fine cytoplasmic granules. In the DI-Syston and Kelthane cultures, most cells showed large cytoplasmic granules around the nuclei. Multinucleated cells and giant cells were also present.

TABLE 5

CHRONIC TOXICITY OF INSECTICIDES IN HELA CELL CULTURES*

| Insecticide | Chronic Toxicity | | | Acute Toxicity $ID_{50}$ |
|---|---|---|---|---|
| | Non-toxic Conc., µg/ml | Minimal Toxic Dose, µg/ml | Tolerated Toxic Conc., µg/ml | Chronic Toxicity MTD |
| Cygon | 2.0 | 20.0 | 100 | 11 |
| Dipterex | 5.0 | 10.0 | 10 | 2.2 |
| Malathion | < .1 | .1 | 20 | 130 |
| DI-Syston | .5 | 5.0 | 10 | 1.8 |
| Chlordane | < .1 | .1 | 10 | 220 |
| Kelthane | .5 | 5.0 | 10 | 4.6 |
| Karathane | 1.0 | 5.0 | 5 | .8 |

* GABLIKS, J. 1965. Proc. Soc. Exp. Biol. Med. **120**: 168.

*Induced Resistance to Insecticides*

To test the insecticide-treated cultures for alterations of cell susceptibility to the previously exposed insecticide, cells were grown without insecticide for 2 or 3 days.

The results of altered cell susceptibility were expressed as the ratios of the $ID_{50}$ or $ID_{10}$ levels of treated cells over the corresponding levels of controls and are summarized in TABLE 6.[7] After long-term exposure for 84 days, the DI-Syston culture was 3.0, chlordane 2.5, and malathion 1.7 times more resistant than the controls. In contrast to the above-indicated cell resistance, the Dipterex and Kelthane cultures were slightly more susceptible.

The resistance of the DI-Syston- and chlordane-treated cells after the removal of the compounds for 3 days was also associated with a growth stimulatory effect by both insecticides upon the treated cells. The results are illustrated in FIGURE 4. In the DI-Syston-treated culture, growth was increased by 88 per cent, compared to 50 per cent inhibition in the controls by the same dose. In the chlordane culture, the dose produced a 16 per cent increase in growth and a 25 per cent growth inhibition in the control culture.

The adaptation of cells to grow in the presence of insecticides was also evident with mouse L-929 strain cells, using Dipterex. The L strain cells originally showed toxic effects to 10 $\mu$g/ml of Dipterex. During an exposure to 10 $\mu$g for 7 days and to 20 $\mu$g/ml for the subsequent 13 days, the cells became completely resistant to Dipterex at 60 $\mu$g/ml and partly resistant at 100 $\mu$g/ml on the 21st day. Accordingly, the Dipterex-treated cells were six times more resistant to Dipterex than the control.

The induced resistance in mammalian cell cultures to insecticides may be associated with several factors: selection of more resistant cell types present in the original culture; induced mutations in the cells followed by selection; and induction of enzyme systems that inactivate the insecticides. None of these possibilities can be preferred on the basis of the experimental data presented.

The theory of enzyme induction is plausible, since it is known that insecticides stimulate hepatic drug-metabolizing enzymes in the rat. The growth-stimulatory effect of DI-Syston and chlordane upon previously treated cells also suggests a certain dependence of previously treated cells on both insecticides—a phenomenon similar to the well-known dependence of certain antibiotic-resistant bacteria to antibiotics.

*Altered Susceptibility to Poliovirus and Diphtheria Toxin*

Since the chronic exposure of cells to insecticides indicated some alterations in cell physiology, we tested these cells for possible changes in their susceptibility to

TABLE 6

RESISTANCE OF HELA CELL CULTURES TO INSECTICIDES AFTER 84-DAY EXPOSURE*

| | Resistance | |
|---|---|---|
| | $ID_{50}$-treated | $ID_{10}$-treated |
| Insecticide | $ID_{50}$ Control | $ID_{10}$ Control |
| Dipterex | .8 | .7 |
| Malathion | 1.1 | 1.7 |
| DI-Syston | 2.2 | 3.0 |
| Chlordane | 1.3 | 2.5 |
| Kelthane | .7 | .7 |

* GABLICKS, J. 1965. Proc. Soc. Exp. Biol. Med. **120**: 168.

such other biologically active agents as mammalian viruses and bacterial toxins. One group of the insecticide-treated HeLa cells was infected with poliovirus, and another group was submitted to reaction with diphtheria toxin.

On the 77th day at the 12th passage, the insecticide-treated cells were subcultured in order to test the cell susceptibility to poliovirus infection. The subcultures for the diphtheria toxin tests were derived on the 108th day, using the 16th passage cultures. For both tests, the subcultures were grown without insecticides. After 2 or 3 days, when cell monolayers had formed, the cultures received fresh medium with poliovirus Mahoney strain, type I, or diphtheria toxin as indicated. Poliovirus titration tests were performed as described by Merchant *et al.*,[20] and the toxin titration tests were performed as described by Gabliks and Solotorovsky.[21]

All insecticide-treated HeLa cultures were susceptible to the lethal infection of poliovirus, but the virus infectivity titers varied in the treated cultures. The differences are listed in TABLE 7.[8] Cygon-, Dipterex-, DI-Syston, chlordane-, and Karathane-treated cells degenerated more rapidly than the control cells, and the infectivity titer of these cultures was also increased. The virus titers in the malathion and Kelthane cultures were comparable to that of the control.

The results of diphtheria toxin titration (TABLE 7) showed that the Karathane-treated cells were 10 times more resistant to the action of diphtheria toxin than the normal HeLa cells.[8]

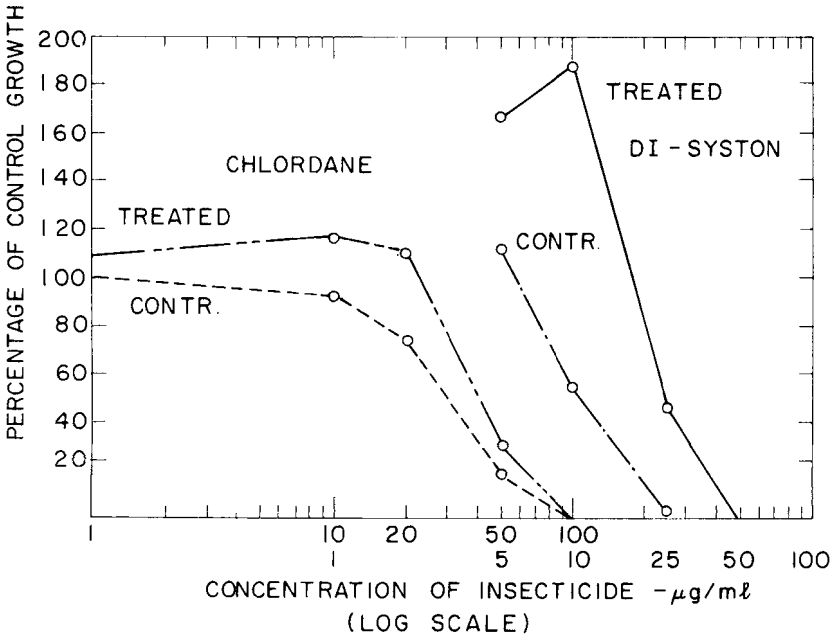


FIGURE 4. Resistance of HeLa cells to chlordane and DI-Syston after previous exposure to the respective insecticides for 84 days. The resistance is indicated by growth curves of the chlordane- and DI-Syston-treated cells and the normal cells in the presence of various concentrations of both compounds. The growth was measured as increase of total cell protein per culture. Low concentrations of both compounds stimulated the growth of the chlordane- and DI-Syston-treated cells.

TABLE 7

SUSCEPTIBILITY OF HELA CULTURES TO POLIOVIRUS AND TO DIPHTHERIA TOXIN AFTER LONG-TERM EXPOSURE TO INSECTICIDES §

| Insecticide | Poliovirus 77 Days Exposure | | Diphtheria Toxin 108 Days Exposure |
|---|---|---|---|
| | Titer $TCID_{100}$ Difference from Control ($log_{10}$) | Degree of Virus Cytopathogenicity* | $TD_{50}$ MLD/ml† |
| None (control) | — | ++ | .5 |
| Cygon | +1.0 | ++++ | .5 |
| Dipterex | +1.0 | ++++ | .5 |
| Malathion | .0 | ++ | .5 |
| DI-Syston | +1.0 | ++++ | .5 |
| Chlordane | +1.0 | ++++ | .5 |
| Kelthane | .0 | + | .5 |
| Karathane | +1.0 | ++++ | 5.0 |

* + = Definite morphogenic changes; ++ = 50% cell degeneration; +++ = 75% cell degeneration; ++++ = 100% cell degeneration.

† MLD titer in guinea pigs.

§ GABLIKS, J. 1965. Proc. Soc. Exp. Biol. Med. **120**: 172.

The greater susceptibility of some of the insecticide-treated cultures to poliovirus infection cannot be explained with the data collected in this study. Considering the alterations associated with increased resistance to insecticides, the increased susceptibility to poliovirus may indicate some additional changes in cell physiology associated with cell susceptibility to virus infections. Although it is not likely that the observed cell alterations with all insecticides are based on impairment of the same mechanism, one possibility may be the inhibition of interferon activity.

One analogous situation, although it does not involve insecticides, has been reported with 20-methylcholanthrene (MC) by De Maeyer and De Maeyer–Guignard.[22] They showed that vaccinia and Sidbis virus plaques were consistently larger in cultures containing 20-MC than in those without it, whereas the yield of virus was not significantly different. This effect was due to the inhibition of interferon production.

The results with diphtheria toxin suggest a possibility of cell mutations induced by Karathane. The possibility of cell mutation is partly supported by the following evidence. Investigations by Gabliks and Solotorovsky[21] indicated that a large variety of primary and established cell cultures derived from susceptible or resistant host species maintain the sensitivity or resistance observed for whole animals. Under certain conditions, generally susceptible cultures may become resistant. Accordingly, Lennox and Kaplan[23] suggested the possible use of diphtheria toxin as a genetic marker for some cultivated cell lines.

In surveying these results the DI-Syston-, chlordane-, and Karathane-treated cells showed altered reactivity in two or more indicator systems and accordingly indicated some definite changes in the physiology of the cells induced by these insecticides. The Karathane-treated cells were more susceptible to poliovirus and more resistant to diphtheria toxin, but were unaltered in susceptibility to Karathane. The DI-Syston- and chlordane-treated cells were more susceptible to poliovirus and more resistant to their respective insecticides. Their growth was stimulated in the presence of the corresponding insecticides, but they were unaltered in susceptibility to diphtheria toxins.

TABLE 8

REPLICATION OF VACCINIA VIRUS IN INSECTICIDE-TREATED RABBIT KIDNEY CELLS

| Insecticide | $ID_{10}$ μg/ml | Test Conc. μg/ml | Growth 7 Days Protein/Culture μg/cult. | Vaccinia Cytopathogenicity Effect* | Virus yield at 48 hr: Infect. $TCID_{50}$ $log_{10}$ | Virus yield at 48 hr: $TCID_{50}$ Culture % Contr. % | Virus yield at 48 hr: Hemagglutination Titer Dil. |
|---|---|---|---|---|---|---|---|
| Control | — | — | 333 "0" hr | | | | |
| | — | — | 1249 | ++++ | 5.0 | 100 | 1/8 |
| Dipterex® | 16.5 | 0.3 | 1231 | ++++ | 4.75 | 56 | 1/16 |
| Malathion | 17.0 | 0.1 | — | ++++ | 4.5 | 32 | 1/16 |
| DDT | < 5.0 | 1.0 | 1283 | ++++ | 5.75 | 562 | 1/32 |

* ++++ = complete destruction of cells.

*Insecticides and Vaccinia Virus Infection*

Alterations in cell susceptibility were also evident with vaccinia virus in primary rabbit kidney cultures. Cultures were exposed to Dipterex, malathion, and DDT at subtoxic levels. After 11 days the cultures were infected with vaccinia virus, and the viral progeny were harvested 48 hr. later. The results are summarized in TABLE 8.[11] The magnitude of cell destruction by vaccinia virus was uniform with all treatments; however, the infectivity titer of progeny varied. For both Dipterex- and malathion-treated cultures, it was somewhat lower, but for the DDT-treated cultures, it was higher than for the control. As further confirmation of the amount of virus yields, a hemagglutination test was performed. The titer for the control was dilution 1/8; for both malathion- and Dipterex-treated cultures, it was dilution 1/16; and for the DDT-treated cultures, it was dilution 1/32.

To prove that the increase in virus yield was due to vaccinia, an attempt was made to neutralize the virus with specific vaccinia antiserum in HeLa cells. Although there was no detectable cytopathogenicity during the first 48 hr of incubation in the presence of vaccinia antiserum, after 96 hr we noticed changes in morphology that progressed with time. The pattern of cytopathogenicity was altogether different from the characteristic plaques given by vaccinia virus. The new plaques appeared as irregularly shaped masses that looked like an agglutination of cell cytoplasm with thin bridgelike connections between.

When the virus pools, harvested from the neutralization test, were passed again into HeLa cells in the presence of antiserum (TABLE 9), the cytopathogenic effect appeared again after 48 hr of incubation. It is interesting to note that after an incubation period of 96 hr, even in the uninfected preparation, cytopathogenicity, which somewhat resembled that obtained from HeLa cultures infected with vaccinia neutralized with antiserum, was evident in one or two isolated spots.

When pools harvested from the second passage with antiserum in HeLa cells were injected into rabbits intradermally, they all gave skin lesions, with the exception of the noninfected culture.

These studies indicate some changes in cell physiology, which affected the replication of vaccinia virus and possibly activated another cytopathogenic agent. This agent appeared to be present in the normal rabbit kidney and may be related to the papova-rabbit kidney vacuolating virus.[24-26]

The identity of the cytopathogenic is still unknown.

TABLE 9

PASSAGE OF VACCINIA VIRUS FROM INSECTICIDE-TREATED RABBIT KIDNEY CULTURE IN HELA CELLS IN THE PRESENCE OF VACCINIA ANTISERUM IN THE RABBIT

| Rabbit Kidney Cultures | | In HeLa Cells with Vaccinia Antiserum | | | | In Rabbit Skin Lesions |
|---|---|---|---|---|---|---|
| Insecticide | Vaccinia | 1st Passage | | 2nd Passage | | |
| | | 48 hr | 96 hr | 48 hr | 96 hr | |
| Control | − | − | − | − | ++ | − |
| Control | + | − | + | + | +++ | + |
| Dipterex® | + | − | + | + | +++ | + |
| Malathion | + | − | ± | + | +++ | + |
| DDT | + | − | + | + | +++ | + |

Key: + Definite cytopathic effect.
++ 50% cell degeneration.
+++ 75% cell degeneration.
++++ 100% cell degeneration.

*Virus Replication as a Parameter of Insecticide Toxicity*

The results of studies with poliovirus and vaccinia virus indicate that alterations in cell metabolism produced by exposure to a compound may influence viral replication, which is dependent upon the normal metabolism of the host cells, and thus provide a new and possibly more sensitive parameter of cytotoxicity.

In additional studies with vaccinia, a DNA virus, and poliovirus (an RNA virus), the magnitude of virus replication was estimated by harvesting cultures 24, 48, and 72 hr after infection. Virus was liberated from cells by freezing, and the yield was estimated by titration of its infectivity in Chang liver cells, using the tube titration method. The infectivity titer was expressed as the tissue culture 50 per cent infective dose ($TCID_{50}$) at any particular time.

The cytotoxicity of insecticides alone was estimated in parallel sets of cultures by microscopic examination of cells to determine morphologic toxicity and by determination of growth inhibition. Growth was measured as the increase of cell number and protein per culture.

The comparative effects of DDT on human liver Chang cells are summarized in FIGURE 5.[9] In the presence of 30$\mu$g of DDT per ml of medium, no cytotoxicity was detectable by microscopic examination. However, the growth curves based on cell count indicated progressive inhibition of growth. At 24 hr it was reduced 15 per cent; at 48 hr, 31 per cent; and at 72 hr, 43 per cent.

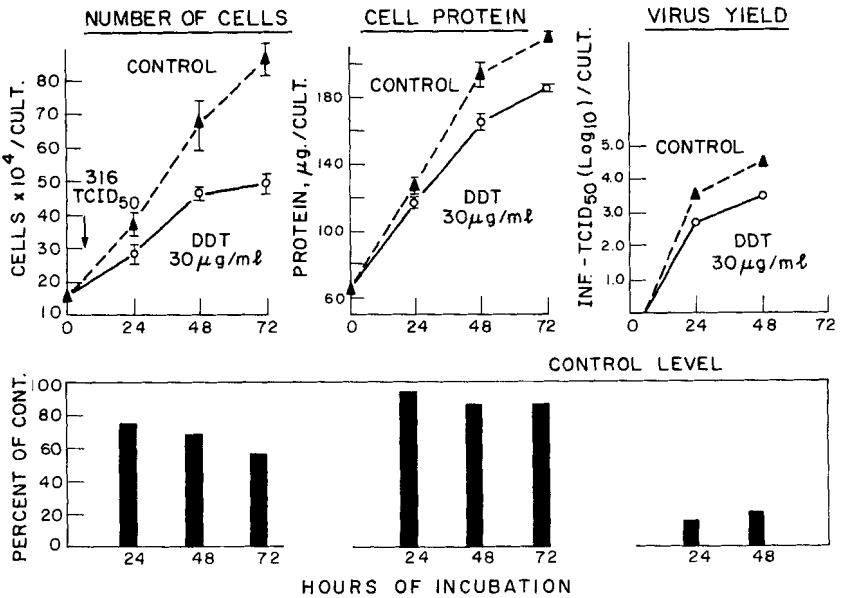


FIGURE 5. Effect of DDT on the growth of human Chang liver cells and on vaccinia virus replication. Cells were exposed to 30 $\mu$g of DDT for 6 hr and then infected with vaccinia virus. The differences in cell growth (measured by cell counts and cell protein determination) and in virus replication (measured as infectivity titers of progeny) are expressed as percentages of their controls on the lower part of the graph. The vertical lines on growth curves indicate the standard deviations of the results. (*From* Gabliks, J. 1967. Arch. Environ. Health 14: 698–702.)

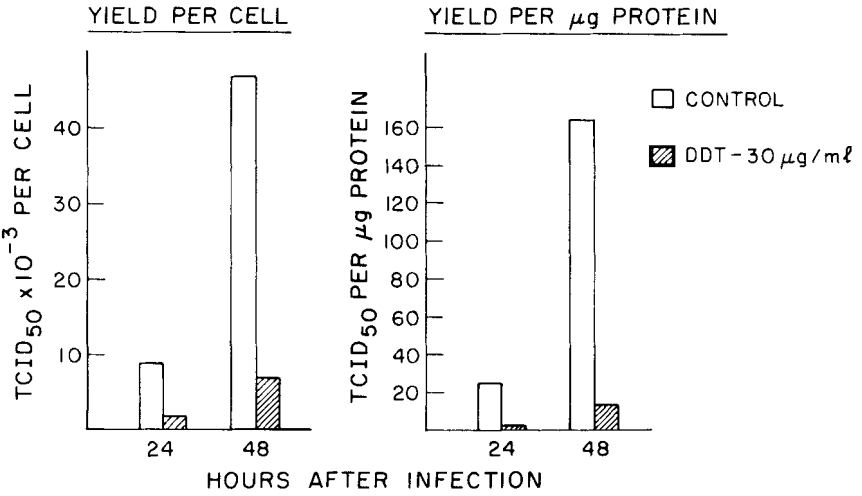


FIGURE 6. Vaccinia virus replication in the DDT-treated human Chang liver cells, expressed as $TCID_{50}$ yields of virus progeny per individual cell and per $\mu$g of cell protein. The virus yield in the DDT-treated cell is reduced. (Gabliks, J. 1967. Arch. Environ. Health **14**: 698–702.)

The progressive reduction of cell growth is also evident from the growth curves based on the protein per culture.

The replication of virus is indicated on the right side of FIGURE 5. In the DDT-treated cultures, the infectivity titer of progeny was 0.8 to 1.0 logarithm lower than in the control.

The results show that DDT, at a concentration that inhibited the growth only 8 to 15 per cent, reduced the virus replication more markedly; i.e., up to 86 per cent.

In order to evaluate the possibility that the reduction of virus yield is not due to the lower number of cells present in the DDT-treated cultures, we calculated the amount of infectious virus released per individual cell or per $\mu$g of protein present in the DDT-treated cultures. FIGURE 6 shows that, during the first 24 hr, the DDT-treated cell released only 18 per cent of the virus released by the control cell. At 48 hr the yield was increased about five times in the normal cells and about four times in the DDT-treated cells, but it was still only 13 per cent of that in the normal cell. The same effect is also evident when the virus yield is expressed per $\mu$g of cell protein, as shown on the right side of FIGURE 6.[9]

Using the same methods as outlined with DDT, we tested chlordane, Kelthane, Dipterex, malathion, and Karathane listed in TABLE 10.[9] At the indicated concentrations of these insecticides, which are several times below their growth inhibitory $ID_{50}$ levels and which did not induce any observable morphologic cytotoxicity, the infectivity titers of progeny were reduced 0.2 to 1.3 logarithms. This reduction in total virus yield per culture corresponded to 47 per cent for Kelthane, the most effective compound.

In a summary of all tests conducted with vaccinia (a DNA virus), the results

TABLE 10

EFFECT OF INSECTICIDES ON VACCINIA VIRUS REPLICATION IN HUMAN CHANG LIVER CELL CULTURES*†

| Insecticide | Conc. μg/ml | Toxicity of Compound | Cell Protein % of Control % | Virus Yield $TCID_{50}$: Titer Differ. from Control $log_{10}$ | $TCID_{50}$/ Culture % of Control % | $TCID_{50}/\mu g$ Cell Prot. % of Control % |
|---|---|---|---|---|---|---|
| *Organochlorine Compounds* | | | | | | |
| DDT | 30.0 | ± | 85 | −0.7 | 20 | 10 |
| Chlordane | 5.0 | 0 | — | −0.8 | 16 | — |
| Kelthane® | 1.0 | 0 | — | −1.3 | 5 | — |
| *Organophosphorus Compounds* | | | | | | |
| Dipterex® | 10.0 | 0 | — | −1.0 | 10 | — |
| Malathion | 10.0 | 0 | — | −0.8 | 20 | — |
| *Dinitrophenol Compounds* | | | | | | |
| Karathane® | 0.1 | 0 | 95 | −0.2 | 63 | 94 |

* Incubated 48 hr.
† GABLIKS, J. 1967. Arch. Environ. Health 14: 698–702.

indicate that all insecticidal compounds tested inhibited the replication of vaccinia virus.

In poliovirus experiments, the virus solution was added 24 hr after the addition of insecticides.

FIGURE 7 summarizes the effects of DDT at 20 and 40μg levels per culture.[9] The yield of infectious virus released per individual cell was increased 37 and 90 per cent, respectively. Similarly, the yield per μg of protein was also increased 15 and 47 per cent, respectively.

TABLE 11 summarizes the effects of other insecticides on poliovirus replication.[9] At the indicated levels, the insecticides did not induce any detectable morphologic cytotoxicity, but the total protein per culture was reduced from 8 to 13 per cent. The total virus yield in the chlordane-treated culture was reduced 68 per cent and in the malathion-treated cells, 82 per cent. In contrast to the action of these two insecticides, the Kelthane-treated cultures gave about 4 times more virus, and the Karathane-treated cells, 18 times more virus. The same relative magnitude of virus yield is also evident when the yields are expressed per μg of cell protein present in those cultures.

An increased yield of poliovirus was also evident, using HeLa cells previously exposed to Karathane for 77 days.[8]

The presented results support our hypothesis that the virus replication test may be useful as an indicator system for detection of alterations in cell metabolism induced by subtoxic concentrations of insecticides or other chemicals.

The altered reactivity of cells to viruses in the presence of insecticides may have significance with respect to the possibility that similar changes take place in animals during chronic exposure to insecticidal residues present in our food supply.

In addition to a possible effect on the pathogenesis of the well-recognized viral diseases, the state of some latent plant or animal viruses may also be altered.

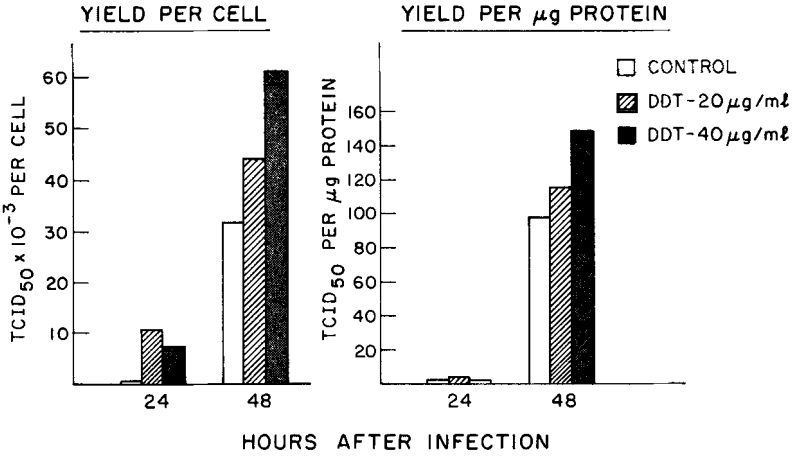


FIGURE 7. Poliovirus replication in the DDT-treated human Chang liver cells, expressed as $TCID_{50}$ yields of virus progeny per individual cell and per μg of cell protein. The virus yield in the DDT-treated cell is increased. (Gabliks, J. 1967. Arch. Environ. Health **14**: 698–702.)

TABLE 11

EFFECT OF INSECTICIDES ON POLIOVIRUS REPLICATION IN HUMAN CHANG LIVER CELL CULTURES*†

| Insecticide | Conc. μg/ml | Toxicity of Compound | Cell Protein % of Control % | Virus Yield $TCID_{50}$: Titer Differ. from Control $log_{10}$ | Virus Yield $TCID_{50}$/Culture % of Control % | Virus Yield $TCID_{50}$/μg Cell Prot. % of Control % |
|---|---|---|---|---|---|---|
| *Organochlorine Compounds* | | | | | | |
| DDT | 20.0 | ± | 87 | 0.0 | 100 | 119 |
| Chlordane | 5.0 | 0 | 94 | −0.51 | 32 | 35 |
| Kelthane® | 1.0 | 0 | 89 | +0.53 | 430 | 480 |
| *Organophosphorus Compounds* | | | | | | |
| Malathion | 10.0 | 0 | 93 | −0.75 | 18 | 19 |
| *Dinitrophenol Compounds* | | | | | | |
| Karathane® | 0.1 | 0 | 92 | +1.25 | 1800 | 1900 |

* Incubated 48 hr.
† GABLIKS, J. 1967. Arch. Environ. Health **14**: 698–702.

We have reported the cytotoxicity of 17 insecticides from three chemical classes to cell cultures derived from man, rabbit, and mouse. The relative toxicity *in vitro* correlated only in some cases with known information on animal toxicity, indicating the important role of metabolic factors present in the intact organism that are absent from the relatively simple *in vitro* system. Chronic exposure of cell cultures to subtoxic levels resulted in some cases in an increased resistance to

the acute toxic effects and also to a growth-promoting effect of subtoxic concentrations.

Changes in susceptibility of cell cultures to the toxic effects of diphtheria toxin and virus infection may be promising as sensitive parameters of subtle effects on the physiology of insecticide-treated cells and possibly even as indicators of mutation.

The usefulness of the *in vitro* animal cell cultures for the study of complex biochemical mechanisms without interference from the influences of the many physiological systems in the intact animal is clearly evident. We should like to emphasize the potential value of studies with a wide variety of human and animal cultures, particularly primary cultures, for providing information that would be helpful in predicting human susceptibility to the toxic action of compounds. Such data, together with comparative metabolic and tissue distribution data in man and animals, may help us bridge the gap between the results of animal experimentation and their more reliable application to human situations.

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