result from an increase in capillary permeability since total protein concentration in this group is markedly reduced.

Furthermore, if there is initially an increase in flow of globulins from the pleural space in the chymotrypsin-treated rats this should result in a change in the A/G ratio of the lymph. The drop in the A/G ratio of the thoracic duct lymph in the enzyme-treated group (Table III) further supports the contention that chymotrypsin increases lymphatic drainage from the inflamed pleural space. If chymotrypsin or some substance released by chymotrypsin caused a generalized increase in lymphatic drainage, we would expect to find an elevated A/G ratio since it would be expected that there would be an increase in the smaller, more abundant albumin materials in the lymph. The drop in protein concentrations of the thoracic duct lymph (Table III) is likewise explainable on the basis of increased lymph drainage from the pleural space. Since the irritant was non-protein in nature it would dilute the proteins in the lymph if it could enter the lymph.

These results indicate that chymotrypsin or some substance released by chymotrypsin increased lymph drainage from the inflamed pleural space without a concomitant increase in capillary permeability. This could result from enzymatic hydrolysis of the proteins which caused lymph stasis in the irritated animals.

Summary. The activity of chymotrypsin, plasmin and trypsin in serum and edema fluid was determined manometrically. It was found that only chymotrypsin, of the enzymes tested, could be found in an active form in these fluids. The volume of edema and its protein concentration were compared in pleural irritated and irritated-chymotrypsin treated rats. Flow rate and protein composition of thoracic duct lymph were also compared between both groups. Changes in lymph and edema in the 2 groups indicate that chymotrypsin increases the rate of flow of lymph from the pleural space without a concomitant increase in capillary permeability. No attempt was made to describe the mechanism responsible for this increase in lymph flow.

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Responses of Cell Cultures to Insecticides. I. Acute Toxicity to Human Cells.* (30475)

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The increased use of agricultural pesticides poses a continuous challenge to scientists concerned with setting of residue tolerances that will be consistent with the safety of the food supply. Although the toxic manifestations of

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insecticides in many mammalian species are well known, the present knowledge of cell responses to insecticides is very limited. Knowing that some members of the organochlorine insecticides persist in animal tissues (1,2,3) and, accordingly, cells are continuously exposed to their metabolites, one must consider the possibility that these chemicals might adversely alter some physiological activities of mammalian cells and lead to general pathological conditions.

These considerations prompted studies aimed at determining cell responses during acute and chronic exposures to insecticidal compounds utilizing cell cultures. Studies of cytotoxicity in human and animal cell cultures might be helpful in evaluation of safety by bridging some gaps between the results of animal studies and their application to man. Further, cell reactivity to insecticides may reveal additional effects which may be obscured in animal studies due to the interference of many physiological systems of the host.

The present study deals with the evaluation of the acute toxicity of insecticides to human cell cultures.

Materials and methods. Cell cultures. Cell responses to insecticides were studied in human cell strains, the Chang liver strain derived from normal liver tissue and the HeLa strain derived from a cervical carcinoma. Both cultures were maintained in Eagle's basal medium(4) supplemented with 10% calf serum. In all tests the medium contained 100 units/ml penicillin and 100 μg/ml streptomycin. For cytotoxicity tests cells were suspended in growth medium at a concentration of 100,000 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. All commercial insecticides used in this study were purified compounds. Their chemical names and purity are indicated in Table I.

For cytotoxicity tests the water soluble compounds Dipterex® and Cygon® were dissolved in the growth medium directly. The water insoluble compounds, malathion, DI-Syston®, chlordane, Kelthane®, ovex, dinitro-o-cyclohexylphenol and Karathane® were first dissolved in ethyl alcohol and then diluted in the medium by a 100-fold step, and further with medium as required.

As determined in previous experiments, an ethyl alcohol concentration in the medium below 0.1% was not toxic to the cells. Some organochlorine compounds, Bulan®, DDD, DDT (technical grade), heptachlor, methoxychlor and Perthane® were tested in the medium as fine suspensions which were obtained using a high speed glass homogenizer. Insecticide stock solutions or suspensions were adjusted to pH 7.5 with $NaHCO_3$ or NaOH solutions, when necessary.

Test procedures for cytotoxicity. Toxicity tests were performed in cell monolayer tube cultures. Insecticides were incorporated in the growth medium at 4 different concentrations in triplicate. The treated cultures were then incubated for 24 and 48 hours on a roller-drum at 37°C. The toxic effect of insecticides was evaluated in 2 ways. First, microscopic examination was made of the cells to determine the cytopathogenic effect, which, in general, was characterized by progressive morphological changes leading to the destruction of the cells. Destruction or morphological alteration of 75 to 100% of the cells was classified as a 4+ reaction, of 50 to 75% as 3+, and of 25 to 50% as a 2+ reaction. The lowest concentration of an insecticide which induced a 2+ reaction during the incubation period of 48 hours was expressed as the toxic dose 50% or TD_{50}.

The second method determined the growth inhibition of cells in the presence of insecticides. The growth of the culture was measured by determination of total cell protein since the increase of cell protein per culture is generally proportional to the increase in cell number(5,6). Concentrations of an insecticide which inhibited the growth by 10% and 50% during the incubation period of 48 hours were designated as the inhibitory doses 10% and 50%, *i.e.*, ID_{10} and ID_{50}.

Method for cell protein determination. After a specified incubation period, the insecticide-containing medium was removed from the cultures. The cell monolayers were

TABLE I. Insecticidal Compounds.

Trade name	Chemical name	Purity, %
	Organophosphorus compounds	
Cygon®[1]	0,0-Dimethyl S-(N-methylcarbamoylemethyl)phosphorodithioate	99.8
Dipterex®[2]	0,0-Dimethyl(1-hydroxy-2,2,2-trichloroethyl)phosphonate	100
DI-Syston®[2]	0,0-Diethyl S-(2-ethylthio)ethyl phosphorodithioate	96.9
Malathion[1]	0,0-Dimethyl S-(1,2-dicarbethoxyethyl)phosphorodithioate	99.6
	Organochlorine compounds	
Bulan®[3]	2-Nitro-1,1-bis(p-chlorophenyl)butane	98
Chlordane[4]	1,2,4,5,6,7,8,8-Octachloro-2,3,3a,4,7,7a-hexahydro-4,7-methanoindene	Reference grade
o,p'-DDD[5]	2-0-Chlorophenyl-2-p-chlorophenyl-1,1-dichloroethane	
p,p'-DDD[5]	2,2-bis(p-chlorophenyl)-1,1-dichloroethane	
DDT[5]	1,1,1-Trichloro-2,2-bis(p-chlorophenyl)ethane	Technical p,p'-isomer 77.2
Heptachlor[4]	1,4,5,6,7,8,8-Heptachloro-3a,4,7,7a-tetrahydro-4,7-endomethanoindene	97.8
Kelthane®[6]	4,4'-Dichloro-α-(trichloromethyl)benzhydrol	96
Methoxychlor[7]	1,1,1-Trichloro-2,2-bis(p-methoxyphenyl)ethane	Analytical standard 100
Ovex[8]	p-Chlorophenyl p-chlorobenzenesulphonate	95.14
Perthane®[6]	1,1-Dichloro-2,2-bis(p-ethylphenyl)ethane	100
Prolan[3]	2-Nitro-1,1-bis(p-chlorophenyl)propane	98
	Dinitrophenol compounds	
Dinitro-o-cyclohexylphenol[8]	2-Cyclohexyl-4,6-dinitrophenol	98
Karathane®[6]	2-(1-Methylheptyl)-4,6-dinitrophenylcrotonate	88.4

[1] American Cyanamid Co., Princeton, N. J. [2] Chemagro Corp., Kansas City, Mo. [3] Commercial Solvent Corp., New York. [4] Velsicol Corp., Chicago, Ill. [5] Edcan Laboratories, South Norwalk, Conn. [6] Rohm and Haas Co., Philadelphia, Pa. [7] E. I. DuPont Co., Wilmington, Del. [8] Dow Chemical Co., Midland, Mich.

washed twice with balanced salt solution to remove all traces of protein present in the test medium. The amount of cell protein was determined by the method of Lowry *et al* (7) as modified by Oyama and Eagle for tissue cultures(5). The growth of treated cultures was expressed as the percentage of the control growth. The inhibitory doses ID_{10} and ID_{50} for each insecticide were calculated by the method of Litchfield and Wilcoxon(8) from the results of 4 different concentrations.

Results and discussion. Cytotoxic effects. All insecticides tested induced cytotoxic effects in Chang liver and HeLa cell cultures. The cytotoxicity was made evident by progressive inhibition of cell growth. The growth inhibition was associated with or followed by cytopathic changes in cell morphology and led to destruction of cultures. Although the cytopathic effect varied with individual compounds and cell strains, certain manifestations were general and characteristic for most compounds. The early morphological changes were evident in the cytoplasm which became more granular. With some compounds, *e.g.*, Cygon, DI-Syston, the granulation process was very pronounced and led to accumulation of large cytoplasmic granules. On continued incubation the cell process started to retract toward the cell nuclei, and cells became rounded and detached from the glass. In all cases the morphological alteration process was progressive and based on the test concentration and the length of incubation.

Sensitivity of the testing methods. The results of acute toxicity based on the cytopathogenicity and growth inhibition testing methods showed a close correlation as indicated in Fig. 1. The growth curve of the liver cultures treated with Dipterex at 100 μg/ml indicated a complete growth inhibition and a 4+ cytopathogenicity in the 24-hour tests. At 48 hours the cultures were almost completely destroyed. In the second culture group exposed to 50 μg/ml the growth was inhibited 35% at 24 hours and 50% at 48 hours. The corresponding degrees of cytopathogenicity were classified as 2+ and 3+

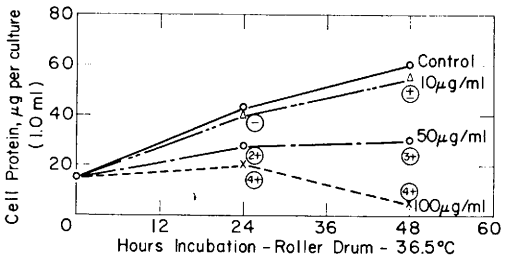

FIG. 1. Effect of Dipterex on growth of human Chang liver cells during 24-hr and 48-hr incubation periods. Cytotoxicity is indicated by scores from — to 4+. Destruction or alteration of 75-100% of the cells is 4+; 50 to 75% is 3+; 25 to 50% is 2+; up to 25% is 1+; slight abnormality of questionable significance is ±; no change is —.

reactions. A lower concentration of Dipterex, 10 μg/ml, reduced the growth very slightly, and the morphological cytopathogenicity test did not show any detectable differences from the controls.

The described cytotoxicity tests with Dipterex were generally characteristic of all insecticides tested and indicated that the growth inhibition test is a more sensitive method than the morphological cytopathogenicity test. All results were reproducible and the variability of results was negligible.

Comparative cytotoxicity of insecticides. Comparison was made on the basis of morphological cytopathogenicity, TD_{50}, and the growth inhibition, ID_{50} or ID_{10} levels. For convenience of presentation, insecticidal compounds are subdivided into 3 groups according to their chemical structures: organophosphorus; organochlorine; and dinitrophenol compounds. The results are summarized in Table II.

(A) The organophosphorus insecticides exhibited a wide range of toxicity. In Chang liver cells, Cygon was the least toxic compound, with an ID_{50} of 170 μg/ml; followed by Dipterex, 35 μg/ml; malathion, 15 μg/ml; and DI-Syston, 5 μg/ml. The same sequence of toxicity was evident in HeLa cells. The TD_{50} levels indicated the same sequence of toxicity. Comparatively, the toxicity of DI-Syston in liver cells is about 34 times that of Cygon, 7 times that of Dipterex and 3 times that of malathion.

Organophosphorus compounds, 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 also appear to be affected by these agents. Inhibition of human serum and liver esterases *in vitro*

TABLE II. Comparative Cytotoxicity of Insecticides to Human Chang Liver and HeLa Cells.

	Liver cells			HeLa cells		
	Cytopathogenicity	Growth inhibition		Cytopathogenicity	Growth inhibition	
	TD_{50}	ID_{50}	ID_{10}	TD_{50}	ID_{50}	ID_{10}
Insecticide	(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.

have been described by Ecobichon and Kalow(9). The negative logarithm of the molar concentrations which inhibited human liver esterases in their system have indicated the same order of toxicity as our cytotoxicity results—Dipterex, 4.82; malathion, 3.00; and DI-Syston, 2.52. These results are in complete agreement with our results of comparative cytotoxicity.

The presented results suggest that the toxicity of organophosphorus compounds may be due to their interference with the activities of esterases present in cultivated cells.

(B) The organochlorine compounds methoxychlor, prolan, p,p'-DDD, o,p'-DDD, DDT, heptachlor, Perthane and Bulan were tested as suspensions in the medium. In Chang liver cultures the toxicity expressed as the TD_{50} ranged from 1000 to 100 μg/ml in the above listed order of increasing toxicity. Their insolubility may be partly associated with the low cytotoxicity. Chlordane, Kelthane and ovex were tested in solutions and their ID_{50} levels were 10 μg/ml, 6 μg/ml, and 5 μg/ml respectively. HeLa cells were 2 to 3 times more resistant than the liver cells with an ID_{50} level for chlordane of 22 μg/ml and for Kelthane of 23 μg/ml.

(C) 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. Dinitro-o-cyclohexylphenol was tested only in liver cultures and its $ID_{50} = 1.0$ μg/ml. In comparison to the least toxic organophosphorus compound, Karathane was 566 times as toxic as Cygon.

With those insecticides which are soluble in the medium, the toxic manifestations are induced with small amounts of compound as compared to those required for animals. In the rat, the LD_{50} for these insecticides ranges from approximately 5000 to 10 mg/kg. Accordingly, the cell culture toxicity titration methods are about 2500 to 3000 times more sensitive than the indicated rat toxicity tests, and therefore can be most useful for studies where only small amounts of insecticidal compounds, their residues and derivatives are available.

This study has shown that insecticides are toxic for cultivated human cells, and cell culture techniques may be useful for studies on the mode of action of insecticides and their bio-transformation. Studies in cultivated cells of animal and human origin also offer possibilities for bridging the gap between the animal results and their application to man. To evaluate this possibility, it will be necessary to accumulate extensive data using substances that have been studied in animals, in man, and in animal and human cells *in vitro*.

Summary. Human Chang liver and HeLa cells were exposed to organophosphorus, organochlorine, and dinitrophenol insecticidal compounds. The acute cytotoxicity was measured during 48-hour incubation and the effects expressed in two ways: the toxic dose (TD_{50}) based on cytopathological effects observed microscopically; and the growth inhibition (ID_{50}) based on inhibition of cell protein synthesis. Results by each criterion closely correlated but the growth inhibition test was generally more sensitive. All insecticides tested were cytotoxic in both cell lines and induced progressive morphological changes leading to destruction of cells. For liver cells the ID_{50} values ranged from 0.3 μg to 170 μg per ml of medium. For HeLa cells the results were approximately the same. Dinitrophenol insecticides were relatively more toxic than organochlorine and organophosphorus compounds.

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Responses of Cell Cultures to Insecticides. II. Chronic Toxicity and Induced Resistance.* (30476)

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Residues of agricultural insecticides are present in the human food supply. Studies in animals have indicated that a regular intake of small amounts of insecticides leads to accumulation of some compounds in animal tissues(1,2,3). DDT and dieldrin have been found in human fat(3). The significance of these findings is not clear but they suggest a potential hazard to human health.

To evaluate the effects of insecticides on human cells, some organophosphorus, organochlorine, and dinitrophenol insecticides have been studied in human cell cultures. In Chang liver and HeLa cells, the insecticides induced cell destruction, and their relative cytotoxicity has been determined(4). As a continuation of the previous studies, this report describes the chronic toxicity of insecticides to human and mouse cell cultures.

Materials and methods. Cell cultures. HeLa cells derived from cervical carcinoma were cultivated in Eagle's basal medium(5) supplemented with 10% calf serum and L-929 mouse cells derived from subcutaneous tissue, in Medium 199(6) supplemented with 10% horse serum. In all tests the media contained 100 units/ml penicillin and 100 μg/ml streptomycin.

For the chronic toxicity tests, cells were suspended in their respective media at 100,000 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.

For testing the insecticide-treated cultures for alterations of cell susceptibility to the previously exposed insecticide, cells were grown without insecticide for 2 or 3 days, and then reexposed to varying concentrations of the compound, as described in the methods of the acute cytotoxicity test(4).

Insecticidal compounds. The insecticides investigated in this study were purified compounds: O,O,-Dimethyl S-(N-methylcarbamoylemethyl)-phosphorodithioate (Cygon®‡); O,O-Dimethyl (1-hydroxy-2,2,2-trichloroethyl)-phosphonate (Dipterex®§); O,O,-Diethyl S-(2-ethylthio) ethyl phosphorodithioate (DI-Syston®§); O,O,-Dimethyl S-(1,2-dicarbethoxyethyl) phosphorodithioate (malathion‡; 1,2,4,5,6,7,8,8-Octachloro-2,3,3a-4,7,7a-hexahydro-4,7-methanoindene (chlordane‖); 4,4'-Dichloro-α-(trichloromethyl) benzhydrol (Kelthane®¶); 2-(1-Methylheptyl)-4,6-dinitrophenyl crotonate (Karathane®").

The methods of treating the cell cultures are the same as described previously(4).

Test procedure for chronic cytotoxicity.

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† With technical assistance of Lucan Anthony.

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§ Chemagro Corp., Kansas City, Mo.
‖ Velsicol Corp., Chicago, Ill.
¶ Rohm and Haas Co., Philadelphia, Pa.