physiological aberrations due to endocrine hormone turnover by induced enzymes may result (Street *et al.*, 1969)].

Therefore, the data presented herein indicate that benzene and methylenedioxyphenyl-containing JHA's do resemble insecticide synergists in their reactions with microsomal systems. They do not appear to have any acute toxicity where mammals are concerned (Smalley, 1972), but one should be wary of long-term effects such as induction.

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A Screen for Pesticide Toxicity to Protein and RNA Synthesis in HeLa Cells

Brian C. Myhr

Suspension cultures of HeLa cells were used in a screen for toxicity based upon an initial biochemical response rather than the usual measurements of growth inhibition. Cells were exposed for 30 min to 350 μg/ml dosages of 30 different pesticides, and the effects on [^{3}H]uridine and ^{14}C-labeled amino acids incorporation into RNA and protein were determined. Dose–response curves for DDT, aldrin, carbaryl, and parathion yielded ID_{50} values that showed this method was as sensitive as 48-hr growth measurements. One-half of the compounds studied had no effect on one or both incorporation activities. Eight pesticides selectively inhibited amino acid incorporation, while only one, chlorpropham, was a selective inhibitor of uridine incorporation. Propham, however, inhibited both processes. The actions of the organophosphorus compounds were as varied as the side chains, but all of the chlorinated hydrocarbons except dieldrin strongly inhibited amino acid incorporation. Uncouplers of oxidative phosphorylation were highly inhibitory.

Heightened concern over the potential health hazards of environmental agricultural chemicals has prompted several recent studies of the toxic effects of a number of insecticides on mammalian cell cultures (*i.e.*, Gabliks and Friedman, 1969; Litterst *et al.*, 1969; North and Menzer, 1970) and on chick embryo cells (Wilson and Stinnett, 1969; Wilson *et al.*, 1968). This use of cell cultures represents an attempt to avoid the expense, long time periods, and the complexity of interactions that apply to the usual animal toxicity studies. Such studies have emphasized measurements of the inhibition of cell growth or qualitative changes in cell morphology caused by exposures to several concentrations of different chemicals over time periods usually ranging from 24 to 72 hr. In this way, relative ratings of cytotoxicity or growth inhibition have been obtained. The interpretation of these ratings has been hampered, however, by the lack of knowledge of the many possible changes in both cellular physiology and the nature of the chemical challenge over the long exposure periods.

The purpose of this paper is to present the results of a screen for chemical toxicity based upon initial cellular responses instead of growth inhibition. Such a screening procedure attempts to indicate the relative sensitivity of a prechosen cellular activity to various added chemicals. Thus, the effects of 30 min of exposure to pesticides on precursor incorporation rates into protein and RNA in HeLa cells are described herein. Organophosphorus pesticides are known to inhibit various esterases in cell cultures (DuBois *et al.*, 1968; North and Menzer, 1970), liver glutamate dehydrogenase (Freedland and McFarland, 1965), and trypsin (Ooms and VanDijk, 1966), while some organochlorine compounds inhibit lipase and hexokinase (Sadar and Guilbault, 1971), yet the effects on protein and nucleic acid synthetic rates in animal cells have been

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largely uninvestigated. Litterst *et al.* (1969) have described initial attempts to measure such effects in HeLa cell cultures and concluded they are generally small and therefore insensitive measures of pesticide toxicity. By employing a different experimental approach and extending the study to a larger number of pesticides, we conclude, instead, that measurements of precursor incorporation into protein and RNA may prove to be useful in the selection of toxic pesticides for further studies of their interaction with cellular physiology. In addition, the method described is directly applicable to primary cultures of differentiated cells as well as established cell lines.

METHODS AND MATERIALS

Cell Culture. Hela S-3 cells were maintained in spinner culture (Bellco Glass flasks) (McLimans *et al.*, 1957) at 37° in Joklik-modified SMEM containing 10% (v/v) undialyzed calf serum, 0.1% pluronic F-68 (Wyandotte Chemical Company) (Swim and Parker, 1960), and 75 units of penicillin G and 50 μg of streptomycin sulfate per milliliter. The cells, calf serum, and medium in dry form were supplied by Grand Island Biological Company. The stock spinner cultures were diluted at daily intervals to 3×10^5 cells/ml, and all experiments were carried out with cells in the exponential growth phase with 25–30-hr doubling times. Just prior to use, the required aliquot was removed, centrifuged for 10 min at $300 \times g$, and resuspended in fresh warm medium at 40×10^5 cells/ml.

Materials. Dimethoate (Cygon, 99.3% analytical standard) was a gift from American Cyanamid Company, Princeton, N. J., and trichlorfon (Dylox, 95%) was a gift from Chemagro Corporation, Kansas City, Mo. All other pesticides were supplied at greater than 99% purity in a kit from Chem Service, Inc., Media, Pa. The names of the pesticides screened in this study are given in Table I. Stock solutions were prepared at 20 mg/ml in dimethylsulfoxide (DMSO).

^{14}C-Labeled amino acid hydrolysate (100 μCi/ml, Schwarz mixture) and [^{3}H]uridine (26 Ci/mM; 0.5 mCi/ml) were from Schwarz/Mann, Aquasol scintillator came from New England Nuclear Corporation, puromycin hydrochloride was from Nutritional Biochemical Corporation, and actinomycin D was obtained from Calbiochem.

Screening Procedure. Each pesticide was equilibrated by gentle shaking in the complete growth medium for 1 hr at 37° prior to use. The dosage was kept constant near 0.4 mg/ml, regardless of solubility, and since 2% DMSO (v/v) was thereby introduced, a control medium was also prepared containing 2% DMSO. Each screening experiment consisted of a series of 12 conical centrifuge tubes to which 4.5 ml of the appropriate medium and 0.1 ml of labeled precursor had been added. Incorporation was initiated by the addition of 0.5 ml of cell suspension (40×10^5 cells/ml), stoppering and mixing, and incubation in a 37° water bath. The final components per tube were 4×10^5 cells/ml, 346 μg of pesticide/ml (except for controls), 1.7% DMSO, and either 0.25 μCi of ^{14}C-labeled amino acid mixture or 5 μCi of [^{3}H]uridine (1×10^{-4} *M*).

The incorporation of label into trichloroacetic acid (TCA) precipitable material in 30 min was used as a measure of the protein and RNA synthetic rates. Four experimentals and four controls were incubated, while another four tubes were terminated immediately to provide a zero time count correction. The tube contents were mixed by inversion every 5 min during the incubation period. Incorporation was terminated by the addition of 5 ml of ice-cold Dulbecco phosphate-buffered saline (PBS), centrifugation at $700 \times g$, and decantation of the supernatant. The control cell pellets were then resuspended in 5 ml of warm pesticide/medium, 5 ml of cold PBS was added, and the cells were centrifuged and washed once more with 10 ml of cold PBS. The other cell pellets were washed once with 10 ml of PBS. This procedure introduces approximately the same amount of residual pesticide in all the cell pellets in order to compensate for any quenching effect on the scintillation counting efficiency. All cell pellets were then vortexed in 2 ml of cold 10% TCA and placed on ice for at least 10 min. If incorporation into protein was to be determined, the TCA-insoluble precipitates were heated to 90° for 15 min to destroy amino acid-charged tRNA. (Compared to unheated controls, about 12% of the insoluble ^{14}C counts were released by this treatment.) The TCA precipitates were collected by centrifugation at $1200 \times g$ and washed three times by centrifugation with 4-ml aliquots of cold 5% TCA.

Table I. Common Names and Chemical Names of the Pesticides Tested

Common name	Chemical name
	Halogenated hydrocarbons
Aldrin	1,2,3,4,10,10-Hexachloro-1,4,4a,5,8,-8a-hexahydro-1,4-*endo*, *exo*-5,8-dimethanonaphthalene
Chlordane	1,2,3,5,6,7,8,8-Octachloro-2,3,3a,4,7,7a-hexahydro-4,7-methanoindene
DDT	1,1,1-Trichloro-2,2-bis(*p*-chlorophenyl) ethane
Dieldrin	1,2,3,4,10,10-Hexachloro-6,7-epoxy-1,4,4a,5,-6,7,8,8a-octahydro-1,4-*endo*, *exo*-5,8-dimethanonaphthalene
Dicofol	1,1-Bis(*p*-chlorophenyl)-2,2,2-trichloroethanol
DBCP	1,2-Dibromo-3-chloropropane
	Organophosphorus compounds
Diazinon	*O,O*-Diethyl *O*-(2-isopropyl-4-methyl-6-pyrimidinyl) phosphorothioate
Dichlorvos	*O,O*-Dimethyl 2,2-dichlorovinyl phosphate
Dimethoate	*O,O*-Dimethyl *S*-(*N*-methylcarbamoylmethyl) phosphorodithioate
Ethion	*O,O,O',O'*-Tetraethyl *S,S'*-methylene bisphosphorodithioate
Malathion	*S*-[1,2-Bis(ethoxycarbonyl)ethyl] *O,O*-dimethyl phosphorodithioate
Mevinphos	2-Carbomethoxy-1-methylvinyl dimethyl phosphate
Parathion	*O,O*-Diethyl *O*-*p*-nitrophenylphosphorothioate
Trichlorfon	*O,O*-Dimethyl (2,2,2-trichloro-1-hydroxyethyl)-phosphonate
	Carbamates and thiocarbamates
Carbaryl	1-Naphthyl *N*-methylcarbamate
Chlorpropham	Isopropyl *N*-*m*-chlorophenylcarbamate
EPTC	Ethyl *N,N*-dipropylthiocarbamate
Pebulate	Propyl *N*-ethyl-*N*-*n*-butylthiocarbamate
Propham	Isopropyl *N*-phenylcarbamate
	Other compounds
Captan	*N*-Trichloromethylthio-4-cyclohexene-1,2-dicarboximide
DCB	*o*-Dichlorobenzene
...	*p*-Dichlorobenzene
Dinoseb	2,4-Dinitro-6-*sec*-butylphenol
Dinocap	2-(1-Methyl-*n*-heptyl)-4,6-dinitrophenyl crotonate
Dichlorophen	2,2'-Dihydroxy-5,5'-dichlorophenylmethane
PCP	Pentachlorophenol
Simazine	2-Chloro-4,6-bis(ethylamino)-*s*-triazine
2,4,5-T	2,4,5-Trichlorophenoxyacetic acid
...	Tetrachlorophenol
Warfarin	3-(1-Phenyl-2-acetylethyl)-4-hydroxycoumarin

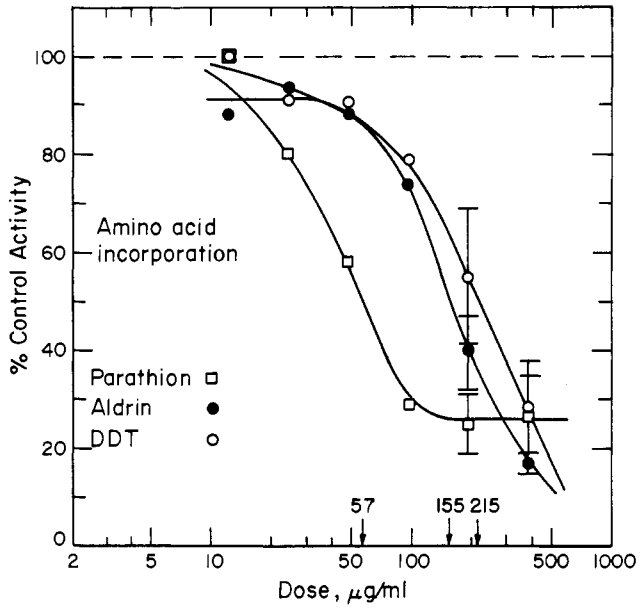

Figure 1. Dose dependency of the effects of parathion, aldrin, and DDT on amino acid incorporation relative to control cultures. The bars show the range of values observed on different days for the dosages at 385 and 192 μg/ml.

The washed TCA pellets were dissolved in 0.5 ml of 1 *N* KOH at 37°, neutralized with 0.5 ml of 1 *N* HCl, and transferred with water washes (2 ml total) to 10 ml of Aquasol for liquid scintillation counting. Counting was performed on Packard Tri-Carb Models 3375 and 2002 with approximately 77 and 26% counting efficiencies (nonoptimal settings) for [^{14}C]amino acids and [^{3}H]uridine, respectively. Typical control counts were 2400 cpm for ^{14}C and 1700 cpm for ^{3}H after small zero time corrections (normally less than 100 cpm). The results were then averaged for each set of four tubes, and the action of the chemical was expressed relative to the control as the percent of incorporation activity remaining.

Dose-Response Curves. Dose-response curves were obtained by diluting pesticide/media prepared near 0.4 mg/ml, as already described, with complete media containing 2% DMSO. The media in these experiments, however, contained 0.12% methylcellulose (Methocel 65 HG, 400 cps, Dow Chemical Company) in place of the 0.1% F68 used in later HeLa cultures. The inhibitory effects at each pesticide concentration were then expressed relative to controls containing 2% DMSO. Each point in Figures 1 and 2 represents the average of at least four experimental tubes, and for the dosages that were tested on different days, the average values and the observed ranges are shown. It is important to note that the concentrations given are actually doses, since DDT, aldrin, and parathion were present as suspensions at the highest levels used. Carbaryl was completely soluble, even though its solubility in water has been reported to be 40 μg/ml (Union Carbide Corporation, 1963). These curves, therefore, yield the dosages in our growth medium at which 50% of the incorporation activity is inhibited (ID_{50}) over the 30-min exposure periods.

RESULTS AND DISCUSSION

Solvent Effects and Culture Conditions. Many of the pesticides are only slightly soluble in water and thus a suitable solvent was required that would have minimal toxicity to the cells. Ethanol at a concentration of 2% (v/v) caused a 31% reduction in amino acid incorporation and 85% inhibition of uridine incorporation, making it unsuitable at this concentration. Dimethylsulfoxide, however, will dissolve almost all the pesticides at stock concentrations of 20 mg/ml, and a 2% concentration caused only 15 and 30% inhibition of precursor incorporation into protein and RNA, respectively. Furthermore, since Diamond (1965) has reported no effects of 1% DMSO on the growth rates of a variety of cell cultures, DMSO was utilized as the carrier solvent in these experiments.

The apparent incorporation activities of the cells vary from day to day, probably due to the inconstancy of culture conditions (pH, nutrient, and metabolic product levels) in spite of pseudo-logarithmic growth, and therefore the response of the cells to a given toxicant may vary daily. In fact, when duplicate experiments were performed on the same day, the incorporation activities relative to controls always agreed within 1–2%, while much larger variations in the effects of some pesticides, especially in the case of aldrin, were observed from one day to the next. Therefore, each pesticide was tested on at least three different days and the results were averaged. On the few occasions when a value markedly different from the others was obtained for a given pesticide, the value was rejected and at least one more test was run.

A systematic investigation of the sources of daily variability has not yet been made, but one important general source is likely to be variations in the exact nature of the chemical challenge to the cells. Pesticide solubility, for example, is often increased greatly by binding to components of the serum additive, and the extent and nature of this interaction depend partly on the equilibration time and the serum composition. Variable inactivation of serum growth-promoting factors may further modulate the observed cell response. Thus, complete removal of the serum component was found to reduce both amino acid and uridine incorporation to 60 and 52%, respectively, of control activities in 10% calf serum. In general, variations in the extracellular environment, including the degree of pesticide availability to the cells, are not closely controlled by working at constant dosage. These considerations also imply that the results reported herein should not be directly compared with other published work in which cells were exposed in media containing different types and concentrations of serum.

Dose-Response Studies. In order to gain some idea of the pesticide dose necessary to provide some selection in a screening program, dose-response curves were constructed

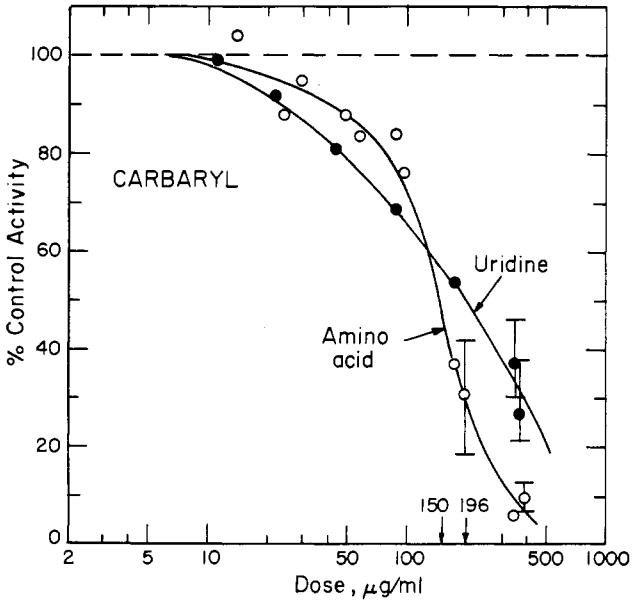

Figure 2. Dose dependency of the effects of carbaryl on amino acid and uridine incorporation. For the dosages repeated on different days, the bars show the observed range of values associated with the plotted average.

for four different types of pesticides: aldrin, DDT, carbaryl, and parathion. These choices also allowed some comparisons of the magnitudes of the derived ID_{50} values with those published for growth toxicity measurements on HeLa cells. The dose dependencies of the effects of aldrin, DDT, and parathion on amino acid incorporation are shown in Figure 1, while the effects of carbaryl on both amino acid and uridine incorporation are given in Figure 2. Only carbaryl had a significant effect on uridine incorporation in the dose range studied. Both DDT and aldrin initially formed very turbid suspensions in the growth medium, but over the course of the hour equilibration period, most of the two pesticides appeared to dissolve. The dose–response curves do not suggest saturation. In the case of parathion, however, the medium remains very turbid and the flattening of the curve above 100 μg/ml probably reflects saturation. The curves for these four compounds suggested that a screen conducted at a pesticide dose between 200 to 500 μg/ml would be adequate for selecting a number of compounds for further study. Since it was desired to first simply select inhibitors (or activators) and then determine the modes of interaction, the screening of insoluble pesticide suspensions did not seem inappropriate. Pesticide suspensions have generally been used for growth inhibition studies.

The ID_{50} values derived from the dose–response curves for amino acid incorporation activity are shown in Table II in which a comparison is made with the limited amount of published data on the effects of pesticides on HeLa cells. It is interesting to note that the procedure used in this study yields ID_{50} values that are not much larger than those obtained from cell growth measurements over 48-hr periods. This result suggests that amino acid incorporation measurements over 30-min exposure periods can be as sensitive a measure of toxicity as cell growth over 48 hr.

Measurements of cell growth in the presence of toxic substances are subject to certain procedural errors that do not apply to incorporation measurements on suspended cells. Thus, substances which cause cells to become less firmly attached to the culture flask, yet which may not interfere significantly with cell growth, will result in a loss of the measured parameter and therefore appear to be highly toxic to growth. Similarly, cell membranes may be weakened by long exposures and become more susceptible to rupture upon the usual trypsinization procedures prior to cell counting. Cell counting itself involves arbitrary decisions on which cells are viable and which should be rejected as irreversibly damaged. Thus, possibly for reasons similar to these, the ID_{50} value reported by Gabliks and Friedman (1965) for disulfoton was significantly smaller than the value of Litterst *et al.* (1969), while the DDT results agreed much more closely (Table II). These differences in growth ID_{50} values suggest that even in the case of carbaryl, where the 30-min amino acid incorporation ID_{50} was five times the growth ID_{50}, the sensitivities of the two cellular responses may not differ as much as indicated.

Although the 30-min amino acid incorporation ID_{50} values can be as small as those derived from growth measurements, and a similar ordering of toxicities exists among the pesticides listed in Table II, such correlations can not, of course, be expected. Trichlorfon, for example, has essentially no effect on either amino acid or uridine incorporation, yet it was reported to be highly toxic to cell growth. Growth inhibition measurements do not directly determine the possible mechanisms of inhibition and, conversely, determinations of initially inhibited biochemical events do not necessarily allow predictions of cellular viability over longer exposure periods. Cellular adaptive mechanisms or the metabolic conversion of pesticides to even more toxic substances are events which are minimized with only 30-min exposures. On the other hand, in the absence of effective cellular adaption or detoxification processes, pesticides which interfere with protein and/or nucleic acid synthesis will certainly interfere with cell viability and growth rates.

Table II. Comparison of Effects of Insecticides on Growth and Protein Synthesis in HeLa Cells

	ID_{50} values, μg/ml		
Insecticide	48 hr[a]	48 hr[b]	30 min[c]
Aldrin	66		155
Dieldrin	250		N.I.
DDT	150[d]	100	215
Malathion		13	V.I.[e]
Trichlorfon		22	N.I.[e]
Parathion	43		57
Diazinon	44		V.I.
Disulfoton	60	9	
Dimethoate		220	N.I.
Carbaryl	30		150 (196)[f]

[a] Values obtained by Litterst *et al.* (1969) from cell counts after 48-hr exposure to insecticide. [b] Values obtained by Gabliks and Friedman (1965) from total cellular protein content after 48-hr exposure. [c] Values obtained by our method with 30-min exposures. [d] In a later publication, Litterst and Lichtenstein (1971) give an ID_{50} value 55 μg/ml for DDT. No reason for this discrepancy was given. [e] V.I., very inhibitory, but ID_{50} values not determined; N.I., not inhibitory. See Table III for effects on precursor incorporation. [f] ID_{50} value for carbaryl effect on uridine incorporation.

Pesticide Screening Results. The results of applying the amino acid and uridine incorporation screens to a larger number of pesticides at dosages near 350 μg/ml are shown in Table III. The pesticides have been arranged into groups which have no effect, those which selectively inhibit either amino acid or uridine incorporation, and those which inhibit both activities. Compounds causing from 25 to 30% inhibition have been somewhat arbitrarily regarded as nonsignificant inhibitors. Of the 30 pesticides tested, one-half had no significant effect on either one or both incorporation activities, while the other 15 compounds inhibited both activities to various extents. Eight pesticides selectively inhibited amino acid incorporation, while only one, chlorpropham, has to date been found to selectively inhibit uridine incorporation. These results clearly show that 30-min incubations at dosages of 350 μg/ml are sufficient conditions to select pesticides which interfere in some manner with either one or both incorporation activities.

The rapidity of action of a well-known inhibitor of protein synthesis, puromycin, was demonstrated in preliminary experiments in which 90% of the total decrease in incorporation rate of leucine into protein occurred within the first 30 min of exposure. Also, as shown in Table III, puromycin at 1.9 μg/ml selectively inhibited amino acid incorporation. Rapid inhibition of RNA synthesis was demonstrated by the use of actinomycin D (0.2 μg/ml), which caused a 34% reduction in uridine incorporation and had no effect on protein synthesis. Thus, the 30-min exposure period seemed sufficiently long to detect significant inhibitors of RNA and/or protein synthesis. The effect of longer exposure periods on the screen selectivity was not investigated.

A direct comparison of the screen results with published data can be made for warfarin and captan. Warfarin is an anticoagulant rodenticide that reportedly has a variety of effects on protein and RNA synthesis (Bernacki and Bosmann, 1970). Thus, it stimulates protein synthesis in isolated rat liver mitochondria but has no effect on rat brain mitochondria or liver microsomes. In L5178Y mouse leukemia cells, however, RNA, DNA, protein, and glycopro-

Table III. Effects of Pesticides at 350 μg/ml on Amino Acid and Uridine Incorporation Relative to Unexposed HeLa Cells

Compound	% incorporation activity[a]	
	[^{14}C]amino acids	[^{3}H]uridine
Compounds having no effect		
Dimethoate	81 ± 1[b]	100 ± 9
Trichlorfon	76 ± 3[b]	81 ± 3
Dichlorvos	73 ± 5	77 ± 6
Mevinphos	93 ± 4	94 ± 4
Simazine	92 ± 1	99 ± 3
Dieldrin	95 ± 5	100 ± 3
Warfarin	99 ± 1	95 ± 6
Selective inhibitors of uridine incorporation		
Actinomycin D (0.2 μg/ml)	99 ± 1	66 ± 18
Chlorpropham	83 ± 8	42 ± 8
Selective inhibitors of amino acid incorporation		
Puromycin (1.9 μg/ml)	57 ± 6	93 ± 3
Dicofol	2 ± 1	80 ± 7
Aldrin	20 ± 2	72 ± 12[c]
Parathion	26 ± 6	73 ± 9
Diazinon	8 ± 5	70 ± 17
Ethion	11 ± 6	118 ± 3
Dinocap	19 ± 6	89 ± 2
Inhibitors of incorporation of both precursors		
DDT	16 ± 4	66 ± 5
Chlordane	5 ± 1	43 ± 6
Carbaryl	10 ± 3	32 ± 8[c]
EPTC	4 ± 2	61 ± 3
Pebulate	4 ± 1	64 ± 5
Propham	43 ± 6	51 ± 11
Malathion	25 ± 14[c]	31 ± 6
2,4,5-T	18 ± 1	60 ± 5
Dinoseb	5 ± 1	13 ± 2
Captan	2 ± 1	1 ± 1
PCP	4 ± 1	5 ± 2
Tetrachlorophenol	3[d]	7 ± 1
DCB	2 ± 1	2
p-Dichlorobenzene	0	11 ± 1
DBCP	10 ± 1	36 ± 12
Dichlorophen	1	3 ± 1

[a] The error terms are Students' standard deviations, $(\Sigma d^2/n - 1)^{1/2}$, computed on three or more values. [b] Incorporation values were obtained at 890 μg/ml in earlier experiments. [c] Seven or more determinations. [d] *Single determination.*

tein synthesis were strongly inhibited. Bosmann and McMinn (1971) recently reported warfarin to be highly inhibitory to all these processes in monolayers of HeLa S-3 cells. Uridine and leucine incorporation were inhibited by 40 and 55%, respectively, in 30-min exposures to a dose near 310 μg/ml. In contrast, warfarin did not inhibit either event in our screen. Warfarin is not soluble in DMSO and therefore was added as a fine suspension to the growth medium in which almost none appeared to dissolve. Bosmann and McMinn apparently converted warfarin to its sodium salt, which is freely soluble in alkaline solution, and therefore may have achieved a much higher percentage of dissolved warfarin in their tests. Warfarin in the particulate state does not appear to be available to inhibit the cells in the 30-min exposure period, even though its high lipid solubility would lead one to expect adsorption to the cell membrane.

Captan is an agricultural fungicide that apparently reacts nonspecifically with cellular sulfhydryl groups, thereby inhibiting many enzymatic reactions. Gale *et al.* (1971) have recently studied in some detail its effects on protein, RNA, and DNA synthesis in Erlich ascites tumor cell suspensions in mice and have observed inhibitory actions in agreement with those given in Table III. Thus, for 20-min exposures at doses exceeding 30 μg/ml, precursor incorporation into all three macromolecular classes was completely inhibited.

The different exposure conditions and dosage levels permit only qualitative comparisons of these results with those of Litterst *et al.* (1969). These workers exposed HeLa cell monolayers to various insecticides at either 10 or 125 μg/ml for 4 hr, and then measured the incorporation of [^{14}C]-leucine, [^{3}H]uridine, or [^{14}C]thymidine during an additional 30-min period. None of the pesticides tested showed any significant effect at the 10 μg/ml level, except possibly DDT, which was reported to inhibit uridine incorporation by 30%. Our results are qualitatively in agreement, since the dose–response curves for DDT, aldrin, parathion, and carbaryl (Figures 1 and 2) show virtually no effect at 10 μg/ml. However, as the dosages are increased, some differences begin to appear. Parathion was reported to still have no effect at 50 μg/ml on any of the incorporation parameters, while 44% inhibition of amino acid incorporation was observed in our 30-min exposure period. In the opposite direction, aldrin at 125 μg/ml was less inhibitory in 30 min (39% inhibition) than over 4.5 hr (66% inhibition). Also, DDT and aldrin had only small effects on uridine incorporation into RNA in our screen as shown in Table III, yet the inhibition reported by Litterst *et al.* (1969) for DDT and aldrin (49 and 34% inhibition, respectively) at 125 μg/ml could lead one to expect significant inhibition at 350 μg/ml in 30 min. These differences in experimental results suggest that other events, such as pesticide metabolism, other modes of action, or increased intracellular pesticide levels are occurring over the additional 4 hr of exposure that cannot be duplicated by higher dosages in shorter times. Gabliks (1965), for example, has demonstrated an adaption by HeLa cells to growth in kelthane and malathion at 100 to 200 times the tolerated dose for unexposed cells.

The effects of various herbicides on precursor incorporation into protein and RNA in excised plant tissues have also been recently described (Moreland, 1967). Thus, Mann *et al.* (1965) examined 23 herbicides for their effects on leucine incorporation into protein by barley coleoptiles and *Sesbania* hypocotyls. Five of these were very inhibitory at 5 μg/ml over the 3-hr exposures (which included a 2-hr labeling period), of which two—chlorpropham and pentachlorophenol—were included in this study. Moreland *et al.* (1969) have similarly examined a series of herbicides which included dinoseb, chlorpropham, 2,4,5-T, and EPTC. Soybean hypocotyl sections were exposed for 6 hr to 0.6-m*M* solutions of these compounds and the concomitant incorporation of [^{14}C]ATP and [^{14}C]leucine was determined relative to controls. Dinoseb and chlorpropham strongly inhibited both incorporation activities, 2,4,5-T was somewhat less inhibitory, and EPTC caused a 42% reduction in ATP incorporation into RNA with little effect on protein synthesis. Except for chlorpropham and EPTC, these cited results are qualitatively similar to the screening results in Table III. Chlorpropham did not inhibit amino acid incorporation in HeLa cells, but EPTC was very inhibitory. Because of the markedly different experimental conditions, however, these differences should not be construed as differences in plant and animal cell responses.

The screening results in Table III really only serve as a useful point of departure for further studies of why some discrimination was achieved. One uncontrolled factor in the screen concerns the amount of pesticide adsorbed to the cell membrane and/or the proportion actually transported into the cells. This probably varies considerably among the tested compounds. A far better comparison of inhibitory activities would be based on the amount taken up by the cells rather than on a constant dosage. Furthermore, the observed changes in precursor incorporation

rates can be a consequence of at least three general mechanisms of interaction: (1) the transport of the radiolabeled amino acids and/or uridine into the cell is inhibited; (2) enzymatic and assembly processes in the pathways from uridine and amino acids to RNA and protein are affected; and (3) the energy requirements of protein and RNA synthesis are not met as a result of interference with mitochondrial function. Studies are continuing on the rates of uptake of precursors in the presence of certain pesticides selected by the screen and also on the proportion of added pesticide actually associated with the cells.

Concerning interference with the production of energy-rich compounds, Gruenhagen (1971) and Gruenhagen and Moreland (1971) have shown that most of the herbicides previously reported to inhibit precursor incorporation into protein and RNA in plant tissues also markedly lower the endogenous ATP levels. Dinoseb, chlorpropham, and 2,4,5-T were among the herbicides that, at 0.6 m*M* dosages, reduced the ATP levels to 10, 12, and 37% of controls, respectively, over 6-hr exposures. In 30 min, however, 0.6 m*M* chlorpropham reduced ATP in plant tissues by only approximately 13%, so the 30-min exposure of HeLa cells to 1.6 m*M* of chlorpropham (346 μg/ml) may not have had a large effect on the intracellular ATP level, as suggested by the lack of inhibition of amino acid incorporation.

Several tentative observations can be made from the screening results in Table III, despite our lack of knowledge of the inhibition mechanisms involved. These observations may serve as focal points for future studies of structure–activity relationships.

Among the eight organophosphorus compounds tested, the structures of the various side chains seem to determine the inhibitory activity rather than the electrophilic character of the phosphorus atom, which is so important for anticholinesterase activity (O'Brien, 1961). Dimethoate, an $R_2P(S)X$ compound, and trichlorfon, dichlorvos, and mevinphos, all $R_2P(O)X$ compounds, had no effect on either incorporation activity. The action of ethion, a bisdithioate, is particularly interesting because it was the only compound tested which appeared to slightly stimulate uridine incorporation while strongly inhibiting amino acid incorporation. Malathion was the only organophosphorus compound which strongly inhibited both activities. Thus, the actions of these compounds were as varied as the phosphate side chains.

The carbamates and thiocarbamates tested appeared to be generally more inhibitory than the organophosphorus group, with carbaryl, EPTC, and pebulate being especially inhibitory to amino acid incorporation. The structural differences between carbaryl, the only insecticidal carbamate tested, and the herbicides were not manifest in this screen. Chlorpropham is a very interesting compound for further study because of its selective inhibition of uridine incorporation and the observation that removal of the meta-chlorine (yielding propham) destroys this selective action by making the compound equally inhibitory to amino acid incorporation.

With the exception of chlordane, the chlorinated hydrocarbons had no effect on uridine incorporation, yet all except dieldrin were strongly inhibitory to amino acid incorporation. Aldrin and dieldrin form an interesting pair for further studies since epoxidation, which is the first step in the metabolism of aldrin in animal species (Fukuto and Metcalf, 1969), results in the loss of inhibition of amino acid incorporation. Dieldrin, however, is far more toxic to animals than aldrin. It also appears that it is the cyclopentane ring of chlordane that enhances its inhibitory activity in some way, since both aldrin and dieldrin contain the remainder of its molecular structure and neither of these compounds are nearly as inhibitory. Chlordane has been shown to interfere with DNA synthesis in L-5178Y mouse leukemia cells by arresting some process during the G_2 phase of the cell cycle (Brubaker *et al.*, 1970), but no direct interference with any macromolecular synthesis has yet been demonstrated.

The molecular structure of dicofol differs from DDT only in the introduction of an hydroxyl group at the carbon bearing the two *p*-chlorophenyl groups. This small change has the effects of both increasing the toxicity to amino acid incorporation and enhancing the selectivity of this action with respect to uridine incorporation. Both compounds are almost completely solubilized at 350 μg/ml by the growth medium.

Dinoseb is a 2,4-dinitrophenol and a known uncoupler of oxidative phosphorylation in plants and rat liver mitochondria (Hemker, 1964). Therefore, its strong inhibitory action on macromolecular synthesis in HeLa cells is probably due to the loss of endogenous ATP within the 30-min exposure period. The other chlorinated phenols tested were also highly inhibitory and all are known to interfere with ATP production. Dinocap is a 2,4-dinitrophenolic ester reported to be very toxic to HeLa cell growth (Gabliks and Friedman, 1965), yet it acted more like the chlorinated hydrocarbons in that only amino acid incorporation was inhibited.

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