Effects of Insecticides on Growth of HeLa Cells

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Monolayer cultures of HeLa cells were used to study the toxicity of selected organophosphorus (parathion, diazinon, Disyston), chlorinated (DDT, aldrin, dieldrin), and carbamate [Carbaryl, Temik (Union Carbide)] insecticides and some of their potential metabolites. Dosages causing 50% inhibition of cell culture growth (ID_{50}) were determined and the effects of the insecticide on synthesis of nucleic acids and proteins were studied. With chlorinated and carbamate insecticides, ID_{50} values varied inversely with the compound's water solubility. Studies with insecticide metabolites indicated that *p*-nitrophenol and α-naphthol were as toxic as the parent compounds. After 4.5 hours of incubation at 10 p.p.m., only diazinon had a deleterious effect on DNA synthesis and only DDT affected RNA synthesis while Disyston was the only compound affecting protein synthesis. At 125 p.p.m., aldrin and DDT showed significant disruptions on all three variables, while parathion at its ID_{50} dose showed no effect on any variable. Exposure of HeLa cells to aspirin or sodium chloride resulted in effects that were similar to those observed with some of the insecticides.

Widespread use of agricultural chemicals in recent decades has resulted in exposure of the general population to certain amounts of these materials. Analyses of body fat from people throughout the world have shown a small burden of these chemicals (Maier-Bode, 1968), particularly those of the chlorinated hydrocarbon group, which can be traced to residues found in food and in the general environment.

The acute, and in many instances chronic, toxicity of these contaminating chemicals has been established for whole organisms, but more information is needed relative to their toxic effect on individual cells. The elucidation of the effects, and ultimately the mechanisms of action, of these chemicals on human cells could add to our understanding of the possible long term effects of agricultural chemicals on humans.

Tissue culture as a tool for the determination of the toxicity of pesticidal chemicals was first used by Lewis and Richards (1945) when they treated a variety of chick embryo tissues with DDT. Their negative results showed DDT to be nontoxic to their tissue system at concentrations as high as 600 parts per million. Not until the middle of the 1960's, however, did workers use cell cultures in an attempt to study insecticide toxicity. In the first of several related publications, Gabliks and Friedman (1965) determined the insecticidal dose (TD_{50}) that caused in fifty per cent of the cell culture certain alterations in cell morphology such as cytoplasmic granulation. Using both HeLa cells and Chang liver cells, the growth inhibition by ten or fifty per cent (ID_{10} or ID_{50}) was determined for eleven different pesticides by measuring the total amount of protein per culture. Their results indicated that dinitrophenol insecticides such as Karathane were more toxic than either the chlorinated hydrocarbon or organophosphorus insecticides used. In 1967 Gabliks *et al.* (1967) tested the effect of several organophosphorus insecticides on mouse liver cell cultures and found very little correlation with data obtained with human strain Chang liver cells. Malathion, for example, was found to be 100 to 120 times more, and Dipterex 10 to 15 times less toxic to human liver cells than to mouse liver cells.

Wilson and Walker (1966) evaluated the toxicity of malathion and some of its analogs to chick embryo cells by studying the effect of the chemicals on cell reproduction. Johnson and Weiss (1967) published results of their studies of the toxicity of DDA to two strains of human cells, utilizing microscopic observations and growth inhibition measurements to investigate toxicity effects. The toxic effects found (ID_{50} of 25 p.p.m. and total disruption of cell monolayers at 50 p.p.m.) could be prevented by the addition of 25 p.p.m. of mevalonic acid to the growth medium. Chung *et al.* (1967) reported on the ability of DDT or dieldrin to reduce or stimulate, depending on the concentration used, the synthesis of nucleic acid or protein in human strain HeLa cell cultures when used at concentrations of 0.5, 10, or 50 p.p.m. Later (1968) Chung *et al.* reported on the change in synthesis of RNA and protein by subcellular fractions of HeLa cells exposed to these insecticides at the same concentrations.

In the present study, tissue culture was selected as the means of investigation because it provides a homogeneous populations of cells upon which biochemical or physical determinations can be conducted with relative ease. The system provides for the direct analysis of the effects of the test compounds on intact cells, as opposed to tissues or homogenates of cells or of tissues.

The decision to use a strain of human cells as opposed to a strain of chicken or rat cells was related to the great concern over the exposure of human beings to contamination from pesticide residues.

The cells chosen for use in this study were strain HeLa, an established cell line originally isolated from a human uterine cervical carcinoma by Dr. George Gey in 1951 (Gey *et al.*, 1952). They are easily handled and maintained and were selected for use in this study because of the knowledge available concerning the cycle of events that leads to replication of HeLa cells. It was hoped that the toxicity of the test compounds could be related to one of the established steps in the life cycle of the cell.

Research data presented in this paper deal with the effect of several insecticides and some of their potential metabolites on cell growth and on the synthesis of DNA, RNA, and protein in HeLa cell cultures.

METHODS OF PROCEDURE

The following analytical grade insecticides or their potential metabolites were used: aldrin, dieldrin, *p, p'*-DDT, *p, p'*-DDE, parathion, paraoxon, paranitrophenol, diethyl phosphorothioic acid, diazinon, Disyston, carbaryl, α-naphthol, and Temik. Tests were also conducted with sodium chloride (purified U.S.P.) and with aspirin (Kent, U.S.P. commercial). The following analytical grade chemicals or solvents were used:

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88% formic acid, 4% perchloric acid, 0.9% saline, 80, 95, and 100% ethyl alcohol (EtOH), anhydrous diethyl ether, redistilled acetone, redistilled pentane, and purified trypsin.

Origin and maintenance of the HeLa cell strain has been described by Mueller *et al.* (1962). Cells used in this study were handled according to the above procedure, using Eagle's basal medium (Eagle, 1955) (Grand Island Biological Co.) fortified with 10% (v./v.) bovine serum and 30 μg. of penicillin G and 50 μg. of streptomycin sulfate per milliliter of growth medium.

HeLa cells were grown as monolayer cultures in three-ounce prescription bottles at 37° C. in an atmosphere of 5% CO_2 in air. Cells were harvested for counting by treating with 5 ml. of 0.05% aqueous trypsin (Fisher Scientific Co.) for 10 to 12 minutes. Action of the trypsin was stopped by adding an equal volume of nonsterile growth medium, and the cells were then resuspended by gentle repipetting.

Before experiments with insecticides were conducted, the effects of some solvents (redistilled acetone or 95% ethyl alcohol) on the reproduction of HeLa cells were determined. For the purpose 0.05, 0.1, or 0.2 ml. of solvent was injected into three replicates of 48-hour old HeLa cell monolayer cultures by means of a sterile syringe, resulting in a final concentration of 0.5, 1, or 2%. After 24, 48, and 72 hours of further incubation replicate cultures were harvested, total cell number was determined, and growth curves were then established.

Growth Inhibition Studies. To determine the effects of insecticides or some of their potential metabolites on cell growth, 0.5×10^6 cells in 5 ml. of growth medium were planted in each growth vessel. After 48 hours of incubation, this starter medium was replaced with 10 ml. of medium containing serial dilutions of the test compounds, which had been added to the medium in an EtOH solution in such a way that the concentration of the solvent did not exceed 0.5%. After 48 hours of additional incubation viable cells (those adhering to the glass surface) were harvested from the culture flask by trypsinization, and the total cell number was then determined, using a Coulter electronic cell counter. Insecticide-induced inhibition of cell growth was then determined by comparing the total number of cells in insecticide-treated cultures with the total cell number in cultures that had been treated with only EtOH (control). The resultant inhibition as related to the insecticide concentration was then plotted on log-probit paper, resulting in dosage-response curves from which the insecticide concentration that caused a fifty per cent inhibition of cell growth (ID_{50}) was determined. Each point on the resulting curves represents the average of three replicates. The standard deviation for each point was always less than 10% of the mean value except for DDT at 50 p.p.m. (36%) and 500 p.p.m. (42%); for parathion at 10 p.p.m. (22%); and for carbaryl at 10 p.p.m. (20%).

To determine the potential effects of insecticides on cell replication with time, growth curves were established by counting the number of viable cells during a 96-hour exposure of these cells to parathion (50 p.p.m.).

It is important at this point to distinguish between Inhibition as it is used in this study, and Lethal, which is commonly used in conventional toxicity testing with whole organisms. The term Lethal implies that the number of surviving organisms is determined after exposure of a given number of organisms to a test compound for a period of time which is significantly shorter than the life span of the organisms themselves. In tests of the type reported in this paper, a colony of organisms is exposed to the test compound for a period of time corresponding to several generation times of the cell, and the number of cells in the resultant colony is compared with the number of cells in the untreated control culture. In cell culture tests of this type, it is not possible to tell whether the alteration in cell number occurred as the result of an effect on cell replication or whether the test compound exerted a directly lethal action on the cell. It is possible only to determine that the total number of cells in the colony was in some manner altered; no statement can be made regarding how or when this alteration occurred.

Effects of Insecticides on Syntheses of Protein and Nucleic Acids. In preliminary experiments designed to determine the effect of aldrin on various biochemical variables in the cells, 100 μg. of this insecticide was applied in 0.1 ml. of redistilled pentane to the inside flat surface of three-ounce prescription bottles. After tipping the bottle to obtain an even coat of aldrin on the flat growth surface, the pentane was evaporated using a gentle stream of filtered air. Cells (0.5×10^6) were then introduced into the growth vessel as previously described and after 24, 48, or 72 hours of incubation the medium was discarded and the cell layer rinsed twice with 10 ml. of 0.9% saline. The monolayer was then rinsed successively with cold 4% perchloric acid, 80 and 100% EtOH, and anhydrous diethyl ether. After drying, the defatted cell layer was dissolved in 2 ml. of 88% formic acid for 20 minutes. The nucleic acid or protein content of the culture was then determined colorimetrically using the method of Ceriotti (1955) for RNA, Kissane and Robins (1958) for DNA, and Oyama and Eagle (1956) for protein.

In all subsequent experiments, however, insecticides were added to three replicates of 48-hour old cell cultures (1 to 1.5×10^6 cells) in 0.05 ml. of 95% EtOH by means of a sterile syringe. One, two, or four hours after insecticide treatment, one of the following precursors was added to replicate cultures by means of a sterile syringe: H^3-leucine (20 μCi/ml.; 5 Ci/m*M*), H^3-uridine (10μCi/ml.; 10 mCi m*M*), or C^{14}-thymidine (4 μCi/ml.; 1 mCi/m*M*). The cultures were then incubated for 30 minutes after which the medium was discarded and the remaining cell layer rinsed twice with 10 ml. of cold 0.9% saline. The monolayer was then defatted as described above and dissolved in 2 ml. of 88% formic acid for 20 minutes. Aliquots of 0.5 ml. were then added to 10 ml. ANPO (alpha-naphthylphenyloxazole) scintillation mixture for analysis. A model 3003 Packard Tri-Carb liquid scintillation spectrometer was then used to assay the total amount of radioactivity incorporated from the radioactive precursors into the cells of the monolayer during the 30-minute exposure period. These data were corrected for background, counter efficiency, and dilutions and expressed as total disintegrations per minute (d.p.m.) per culture.

RESULTS AND DISCUSSION

Growth Inhibition Studies. Solvent toxicity tests showed that at a concentration of 2%, both 95% EtOH and acetone completely inhibited cell culture growth. The same effect was obtained with acetone at a 1% concentration, while at a concentration of 0.5%, a 25% reduction in colony size still was observed after 48 hours of incubation. Ethyl alcohol, though, showed some decrease in total cell number at 1% but had no apparent effect on culture growth at 0.5%. Based on these results, 95% EtOH was selected to be used as the carrier for all insecticides used in later experiments.

Figure 1 and Table I summarize data obtained when HeLa cells were exposed to serial dilutions of insecticides. While Figure 1 shows some typical dosage-response curves obtained with carbaryl, parathion, DDT, and Temik, Table I sum-

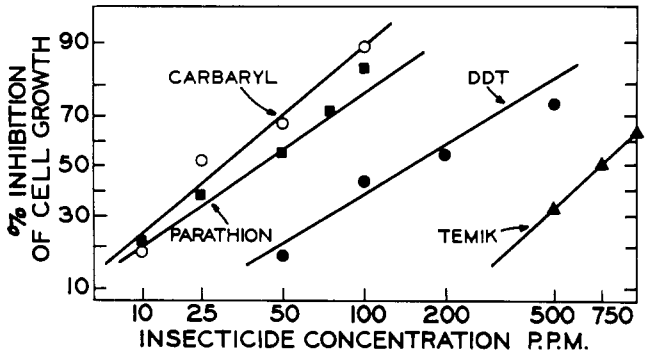

Figure 1. Dosage-response curves obtained after 48-hour exposure of HeLa cell cultures to various concentrations of insecticides

marizes the 50% inhibition levels (ID_{50}) obtained with the eight insecticides used. In general the organophosphorus materials were more toxic than the chlorinated hydrocarbons. The most toxic insecticide tested was carbaryl, while Temik, another carbamate insecticide, was the least toxic material. The value of 150 μg./ml. for the ID_{50} approximates that value found by Gabliks and Friedman (1965) for the toxic dose of DDT to HeLa cells (100 p.p.m.). With chick embryo tissues, however, DDT at 600 p.p.m. was nontoxic as reported by Lewis and Richards (1945).

In considering the differences between the toxicities of the carbamates, carbaryl and Temik, and between the toxicities of the chlorinated materials, aldrin, dieldrin, and DDT, it appears that an inverse relationship exists between the toxicity of these compounds to HeLa cells and their solubility in water. Since these water-insoluble compounds are lipophilic, they would be attracted to the cell monolayer rather than remaining in the growth medium, thus accounting for their increasing effectiveness with decreasing water solubility. The organophosphorus compounds, though more water-soluble than the chlorinated hydrocarbons, appear to be inherently more toxic.

Several potential insecticide metabolites were also tested at various concentrations for their toxicity to HeLa cell cultures. For this purpose HeLa cultures were exposed to some insecticides and some of their potential metabolites, at concentrations that previously had been determined to cause a 50% growth inhibition. In addition, higher concentrations were used. Results indicate (Table II) that DDE was as toxic as DDT at 50 p.p.m. At the 150-p.p.m. test level, both DDT and DDE were injected into 48-hour-old cell cultures, rather than being added as suspensions in newly added medium. This treatment method resulted in an immediate precipitation of the compound and thus may not have produced a representative toxicity, but also at this level (150 p.p.m.) both DDT and DDE exhibited similar levels of toxicity.

Paraoxon, an oxidation product of parathion, was less toxic at 40 p.p.m. than parathion, while paranitrophenol at the same concentration appeared to be slightly more toxic. Both paraoxon and paranitrophenol at extremely high concentrations (250 and 500 p.p.m.) caused almost complete inhibition of cell growth.

It is interesting to observe that, contrary to data with whole animals where oxidation products are generally more toxic than the parent compound and hydrolysis products generally less toxic (Dahm and Nakatsugawa, 1968), results obtained in this study (Table II) seem to indicate that hydrolysis products (α-naphthol from carbaryl and paranitrophenol from parathion) were more toxic than the parent compound to

Table I. Inhibitory Effects of Various Insecticides on Growth of HeLa Cells

Insecticide	ID_{50}[a] (p.p.m.)	LD_{50}[b] (p.p.m.)	Solubility[c] (p.p.m. in water)
Aldrin	66	67	0.01
Dieldrin	250	87	0.1
p,p'-DDT	150	250	0.04
Parathion	43	13	24
Diazinon	44	150–220	40
Disyston	60	12	25
Carbaryl	30	540	40
Temik	750	1	600

[a] Concentration of insecticide in growth medium that caused a 50% reduction in cell number after 48 hours of incubation.
[b] References for LD_{50} data quoted: Aldrin, DDT, Dieldrin (Lehman, 1951); Diazinon, Disyston (Schrader, 1963); Parathion and Carbaryl (Gaines, 1960); Temik (Union Carbide, 1965).
[c] References for water solubilities quoted: Aldrin, Dieldrin (Shell Chemical Corp., 1965); DDT (Robeck *et al.*, 1965); Parathion (Popov, 1956); Diazinon, Disyston (Schrader, 1963); Carbaryl (Union Carbide, 1963); Temik (Johnson & Stansbury, 1966).

Table II. Per Cent Inhibition of HeLa Cell Growth Obtained with Some Insecticide Metabolites

Compound	Concentration (p.p.m.)	% Inhibition[a,b]
p,p'-DDT	50	26[a]
	150	75[b]
p,p'-DDE	50	17[a]
	150	82[b]
Parathion	40	43[b]
Paraoxon	40	17[a]
	250	93[b]
Paranitrophenol	40	55[a]
	250	87[b]
	500	95[b]
Thiophosphoric acid	250	6
	500	23[a]
Carbaryl	30	66[b]
α-Naphthol	25	33[b]
	30	49[b]
	50	61[b]
	100	95[b]

[a,b] Differences observed between control cultures (EtOH treated) and 'compound' treated were significant at the 5% (*a*) or 1% (*b*) level.

HeLa cell monolayers and that the oxidation products (paraoxon from parathion and dieldrin from aldrin) were less toxic.

In time response studies, HeLa cells were exposed for 96 hours to parathion at 50 p.p.m. Inhibitory effects of the insecticide became noticeable only after a 48-hour exposure period, resulting finally in a 60% inhibition of cell growth after an additional 48 hours of exposure. This appears to indicate that the toxicity of parathion is due not to an immediate disruption of some vital mechanism within the cell but rather to an effect which becomes progressively more striking with each passage of the cells through their reproductive cycle.

Effects of Insecticides on Syntheses of Protein and Nucleic Acids. Preliminary experiments using deposits of aldrin on the bottom surface of growth vessels at 10 and 100 μg. per vessel showed no significant effect on cell growth but concentrations of 10, 50, and 100 μg. of aldrin per vessel produced a 3% (significant at the 10% level), 19% (1% level), and 50% (5% level) reduction in cloning ability of the cells. One-hundred micrograms of aldrin per culture vessel showed no significant effects on the total accumulation of protein, DNA, or RNA in HeLa cell monolayers after 24, 48, or 72 hours of treatment, except for a 20% decrease in DNA after 24

Table III. Effect of Various Concentrations of Insecticides, Aspirin, and Sodium Chloride on Synthesis of Nucleic Acids and Protein during a 4.5-Hour Incubation Period

Compound[a]	P.p.m.	DNA D.p.m.[b]	DNA % Ck	RNA D.p.m.	RNA % Ck	Protein D.p.m.	Protein % Ck
I Aldrin	10	1383 ± 241	121	1392 ± 192	81	2952 ± 40	101
II Aldrin	125	270 ± 26	52[d]	982 ± 46	66[d]	824 ± 112	34[d]
III Dieldrin	10	272 ± 48	125	1938 ± 50	111[c]	4056 ± 188	110
I *p,p'*-DDT	10	1158 ± 78	101	1236 ± 12	71[d]	3161 ± 265	108
II *p,p'*-DDT	125	306 ± 2	58[d]	756 ± 12	51[d]	1536 ± 452	64
IV Parathion	10	240 ± 4	97	2348 ± 276	125	7536 ± 168	95
IV Parathion	50	240 ± 8	97	1900 ± 104	102	7952 ± 432	99
III Diazinon	10	280 ± 16	130[c]	1710 ± 162	98	3496 ± 280	95
III Disyston	10	260 ± 20	120	1842 ± 130	105	5084 ± 106	138[d]
V Aspirin	10	452 ± 17	120[c]	2382 ± 38	86[d]	7480 ± 136	107[c]
V Aspirin	125	465 ± 5	124[d]	968 ± 2	35[d]	2683 ± 107	38[d]
V NaCl	10	655 ± 85	175[c]	2630 ± 406	95	8380 ± 497	119[c]
V NaCl	125	600 ± 30	160[c]	2913 ± 87	105	6385 ± 319	91
Control for	I	1130 ± 42		1726 ± 50		2918 ± 143	
	II	524 ± 28		1476 ± 84		2402 ± 626	
	III	216 ± 0		1746 ± 70		3688 ± 232	
	IV	248 ± 12		1868 ± 132		8000 ± 36	
	V	375 ± 25		2762 ± 82		7008 ± 76	

[a] Roman numerals refer to controls, to which each insecticide-treated culture was compared. [b] Total radioactivity per culture expressed in disintegrations per minute found in HeLa cell cultures; average and standard deviation of three replicates. [c,d] Values for treated cultures as a percentage of control cultures (Et-OH-treated) were significant at the 10% (*c*) or 1% (*d*) level.

hours and a 33% decrease in RNA after 48 hours of treatment (significant at 1 and 5% levels). This agrees with other data (Table III) which shows that aldrin at 10 p.p.m. had no effect on either DNA, RNA, or protein synthesis.

Results of experiments designed to demonstrate the potential effects of insecticides, sodium chloride, and aspirin on the incorporation of radioactive leucine, uridine, or thymidine into protein, RNA or DNA, respectively, of HeLa cell cultures are presented in Table III. Data are expressed in total radioactive disintegrations per minute (d.p.m.) per culture and in changes observed relative to controls (EtOH).

At a concentration of 10 p.p.m., the synthesis of DNA was affected only by diazinon, while the other seven insecticides did not produce any changes. However, the same effect observed with diazinon was also obtained with aspirin and NaCl, the latter causing a 75% increase in DNA synthesis. The synthesis of RNA was increased with dieldrin by 11% and decreased with DDT by 29%. Aspirin also decreased this synthesis by 14%. Protein synthesis was affected only by Disyston among the insecticides (at 10 p.p.m.) and produced a 38% increase. However, both aspirin and NaCl also had some increasing effect on protein synthesis, when used at that concentration.

All the above results were obtained after the HeLa cells had been exposed for 4.5 hours to the various chemicals. Toxicity effects were also determined after 1.5 or 2.5 hours of treatment and a certain amount of variability was seen at these times compared with the 4.5-hour results. Aldrin and DDT caused decreases of 39 and 16%, respectively, in the synthesis of DNA after 2.5 hours of treatment, yet this effect was no longer noticeable after 4.5 hours. Similarly, dieldrin caused a 26% increase in DNA synthesis rate after 1.5 hours of treatment, but this effect disappeared completely as the incubation time increased to 2.5 and then to 4.5 hours. DDT caused an initial stimulation of RNA synthesis, which was evident after 1.5 hours of treatment but not at 2.5 hours and which by 4.5 hours of treatment showed a significant (1% level) decrease in the synthesis of this nucleic acid. Disyston caused a significant decrease (5% level) in the rate of RNA synthesis after 2.5 hours but showed no effect after either 1.5 or 4.5 hours. Sodium chloride on the other hand, decreased the synthesis of RNA after both 1.5 and 2.5 (12 and 23%) but showed no effect after 4.5 hours, which perhaps could partially be explained by the large standard deviation of the sodium chloride data after 4.5 hours. After 2.5 hours of treatment, aldrin showed a 28% decrease and dieldrin a 17% increase in protein synthesis, effects that were seen at neither 1.5 nor 4.5 hours of treatment.

Although the test level of 10 p.p.m. is quite high relative to concentrations found in human tissues (except for DDT), it was thought desirable to test the insecticides at concentrations large enough to produce disruptive effects on all variables. Consequently three of the insecticides were tested at concentrations significantly above those which would be encountered in public health situations. The results of these tests are also shown in Table III. Parathion, tested at 50 p.p.m., produced no deviations from control values on any of the three biochemical variables. At a similar concentration, however, (Table I) a 50% inhibition in cell growth was observed after 48 hours of incubation. It is interesting to note that during the first 4 to 5 hours of exposure of HeLa cells to parathion, no effect on cell growth or on synthesis of protein or nucleic acid (Table III) could be observed. After longer exposure, however, (48 hours) differences in cell growth became noticeable and definite inhibition was observed after 96 hours of exposure. Because the life cycle of the cell is approximately 24 hours as predetermined for the conditions in these experiments, the results described above fit into this particular cycle.

DDT and aldrin, when tested at the high dose of 125 p.p.m. produced a 42 and 48% decrease in the synthesis of DNA (significant at 1% level) after 4.5 hours of treatment, while aspirin and sodium chloride at this concentration produced a 24 and 60% increase in the synthesis of this nucleic acid. These latter results were similar to those obtained with lower concentrations (10 p.p.m.) indicating that with increasing concentrations of aspirin or sodium chloride, no increasing effect on DNA synthesis was observed.

The synthesis of RNA in HeLa cells was significantly (1% level) decreased by both aldrin and DDT at concentra-

tions of 125 p.p.m., but it will be noted that aspirin also caused a similar significant decrease of 65%.

The synthesis of protein in HeLa cells was not affected by DDT at 125 p.p.m., while aldrin caused a 56% (1% level) decrease in the incorporation of H^3-leucine which was also noticeable (62% decrease) with aspirin.

Aspirin and sodium chloride were tested in this series of experiments because they are commonly used chemicals. Although the pesticides, when tested at the comparatively high concentration of 10 p.p.m., in some instances showed significant differences from control values, in most cases, either aspirin or sodium chloride or both also caused similar changes.

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