detected is only approximately 10 mol % of the amount of lindane applied, assuming complete conversion. There are two apparent reasons that could cause this low recovery. First of all, there may not have been efficient transfer of all the benzene to the cold trap. This could have been due to an inadequate flow of nitrogen through the sewage vessel. Secondly, and perhaps a more plausible reason, is that the anaerobic organisms in the sewage sludge were utilizing the benzene as a carbon source. For instance, labeled carbon dioxide has been found after the anaerobic incubation of [^{14}C]lindane (MacRae et al., 1967). This implies that lindane, or more probably one of its degradation products (i.e., benzene), is being used as a carbon source.

CONCLUSION

Thus, we have another example of the utility of electrochemical reduction data to predict anaerobic degradation products of organohalogen compounds in the environment. In this instance electrochemistry indicated that the γ-BTC formed during anaerobic degradation should be a transient intermediate and that benzene should be the final reduction product. This was established to be the case by incubating lindane in sewage sludge.

This concept may be carried further. If the anaerobic degradation of compounds is purely a chemical phenomenon, as has been recently suggested by Zoro et al. (1974), one might expect to be able to predict the final degradation products from just a knowledge of the electrochemical reduction potentials. For example, DDT can be anaerobically reduced because its E_{2d} is only −1.240 V (Farwell, 1973). However, its reduction product, DDD, which has an E_{2d} of −2.068 V (Farwell, 1973), cannot be anaerobically reduced because the redox potential associated with the anaerobic conditions is not sufficiently low to promote this reduction. DTE has a reduction potential more anodic than DDT's; thus it is anaerobically reduced, whereas DDE, the reduction product of DTE, is not because its first reduction potential of −1.757 is too cathodic. These data imply that compounds with an E_{2d} more positive than −1.521 V vs. SCE in Me_2SO will be anaerobically degraded and compounds with an E_{2d} more negative than −1.757 V will not be anaerobically reduced. The validity of this generalization will have to be tested by investigating the reduction of more compounds of this type.

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LITERATURE CITED

Beland, F. A., Farwell, S. O., Geer, R. D., *J. Agric. Food Chem.* **22**, 1148 (1974).
Benezet, H. J., Matsumura, F., *Nature (London)* **243**, 480 (1973).
Farwell, S. O., Ph.D. Thesis, Montana State University, Bozeman, Mont., 1973.
Farwell, S. O., Beland, F. A., Geer, R. D., *Bull. Environ. Contam. Toxicol.* **10**, 157 (1973).
Farwell, S. O., Beland, F. A., Geer, R. D., *Anal. Chem.* **47**, 895 (1975a).
Farwell, S. O., Beland, F. A., Geer, R. D., *J. Electroanal. Chem.* **61**, 315 (1975b).
Fukami, H., Kimura, H., Nakazima, M., *Botyu-Kagaku* **18**, 51 (1953).
Glass, B. L., *J. Agric. Food Chem.* **20**, 324 (1972).
Hill, D. W., McCarty, P. L., *J. Water Pollut. Control. Fed.* **39**, 1259 (1967).
MacRae, I. C., Raghu, K., Castro, T. F., *J. Agric. Food Chem.* **15**, 911 (1967).
Mann, C. K., Barnes, K. K., "Electrochemical Reactions in Nonaqueous Systems", Marcel Dekker, New York, N.Y., 1970, pp 212–219.
Orloff, H. D., Kolka, A. J., Calingaert, G., Griffing, M. E., Kerr, E. R., *J. Am. Chem. Soc.* **75**, 4243 (1953).
Plimmer, J. R., Kearney, P. C., Von Endt, D. W., *J. Agric. Food Chem.* **16**, 594 (1968).
Rosenthal, I., Lacoste, R. J., *J. Am. Chem. Soc.* **81**, 3268 (1959).
Schwabe, K., Frind, H., *Z. Phys. Chem. (Leipzig)* **196**, 342 (1951).
Tsukano, Y., Kobayashi, A., *Agric. Biol. Chem.* **36**, 166 (1972).
Zavada, J., Krupicka, J., Sicher, J., *Collect. Czech. Chem. Commun.* **28**, 1664 (1963).
Zoro, J. A., Hunter, J. M., Eglinton, G., Ware, G. C., *Nature (London)* **247**, 235 (1974).

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In Vitro Inhibition of Lactic Acid Dehydrogenase by Insecticidal Polychlorinated Hydrocarbons. 2. Inhibition by Dieldrin and Related Compounds

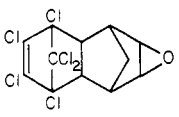
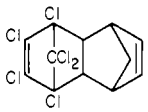
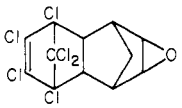
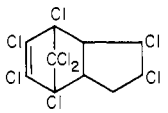
Constance M. Hendrickson[1] and Joe A. Bowden*

Dieldrin and related compounds, including aldrin, endrin, and chlordane, inhibit lactate dehydrogenase (EC 1.1.1.27, crystalline from rabbit muscle). Inhibition is competitive with respect to pyruvate and to NADH; apparent inhibition constants range from 0.02 to 0.06 mM (11 ppm for dieldrin). Levels at which this inhibition occurs are well below the usual lethal in vivo dosages and are comparable to accumulation concentrations. Lactate dehydrogenase occupies a key position in the anaerobic glycolytic pathway of skeletal muscle and in other tissues; impairment of this enzyme's function could result in possible harmful effects on enzyme function.

In previous papers we have described the inhibition of crystalline rabbit muscle lactic acid dehydrogenase by the insecticide Mirex (dodecachlorooctahydro-1,3,4-metheno-2*H*-cyclobuta[*cd*]pentalene) (Hendrickson and Bowden, 1973, 1975a) and DDT (2,2-bis(*p*-chlorophenyl)-1,1,1-trichloroethane (Hendrickson and Bowden, 1975a, 1976b), and have speculated upon the mode and mechanism of the in vitro inhibition of lactic acid dehydrogenase by these neuroactive high molecular weight polychlorinated hy-

Department of Biochemistry, Louisiana State University, Baton Rouge, Louisiana 70803.
[1]Present address: Department of Biophysics, Johns Hopkins University, Baltimore, Maryland 21218.

Table I. Effect of Dieldrin, Aldrin, Endrin, and Chlordane on Lactic Acid Dehydrogenase; Determination of the Apparent Inhibition Constant K_i' and Type of Inhibition[a]

Pesticide	K_i' pyruvate, mM	ppm	Type of inhibition
Dieldrin 	0.03	11.4	Competitive
Aldrin 	0.06	22	Competitive
Endrin 	0.02	7.6	Competitive
Chlordane 	0.05	20.5	Competitive

[a] Experimental conditions: [NADH] = 0.7 mM; [pyruvate] = 0.7–1.4 mM; 1 unit of enzyme in 0.1 M phosphate buffer, pH 7.5 at 25 °C.

drocarbons (Hendrickson and Bowden, 1975b, 1976). In this short paper similar data are presented for the inhibition of lactic acid dehydrogenase by dieldrin (1,2,3,-4,10,10-hexachloro-*exo*-6,7-epoxy-1,4,4a,5,6,7,8a-octahydro-1,4-*endo*,*exo*-5,8-dimethanonaphthalene) and its isomers aldrin and endrin, and by the very similar compound chlordane (1,2,3,4,5,6,7,8,8-octachloro-2,3,3a,4,7,-7a-hexahydro-4,7-methanoindene). The levels of dieldrin used in our studies are comparable with levels found in various species in their natural environment. An excellent review of the environmental data is presented by Stickel (1973) and Edwards (1970).

EXPERIMENTAL PROCEDURE

The method of assay of lactic acid dehydrogenase and the reagents used are described in a previous paper (Hendrickson and Bowden, 1975a). Dieldrin, endrin, and aldrin were gifts of Shell Chemical Co.; chlordane was obtained from Velsicol Chemicals. All were analytical grade standards; purity was checked by melting point determinations. Pesticides were solubilized in 95% ethanol and corrections were made for the effect of ethanol in the assay system as previously described (Hendrickson and Bowden, 1975a). Determination of the type of inhibition and the apparent inhibition constant K_i' was by the method of Dixon (1953) and Lineweaver–Burk (1934).

Time-lag inhibition studies were run under the same conditions as K_i' determinations; however, NADH rather than enzyme was used to start reactions. Visible studies were carried out at room temperature with 1:1 molar ratios of NAD^+, NADH, adenine, or nicotinamide and pesticide. A Beckman DB was used to scan solutions from 340 to 800 nm after 1 week.

A Cary 14 spectrophotometer was used for ultraviolet studies. Pesticide solutions were in ethanol and mixtures

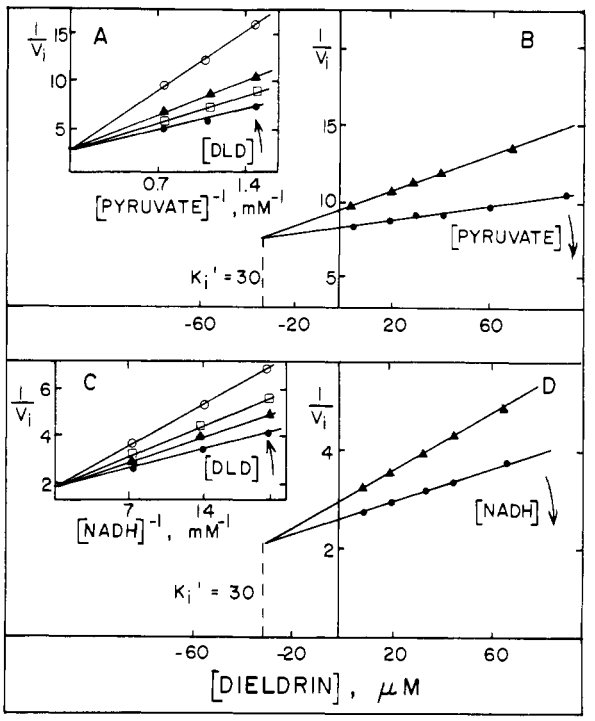


Figure 1. The effect of dieldrin on lactic acid dehydrogenase. (A and B) Effect with respect to pyruvate: (A) Lineweaver-Burk (double reciprocal plot); (B) Dixon (single reciprocal plot). Experimental conditions: [NADH] = 0.7 mM; [pyruvate] = (▲) 0.66 mM; (●) 1.3 mM; [dieldrin] = (●) 0 mM; (□) 0.02 mM; (▲) 0.04 mM; (○) 0.09 mM. The reaction was run in 0.1 M phosphate buffer at pH 7.5, at 25 °C. Abbreviations: dieldrin = DLD. (C and D) Effect with respect to NADH: (C) Lineweaver-Burk; (D) Dixon. Experimental conditions: [pyruvate] = 1.3 mM; [NADH] = (▲) 0.07 mM; (●) 0.14 mM; [dieldrin] = (●) 0 mM; (▲) 0.02 mM; (□) 0.03 mM; (○) 0.04 mM. Conditions same as A–B.

of nucleotide and pesticide were phosphate buffer–ethanol (70:30, v/v). Mixtures of dieldrin with adenine, nicotinamide, NAD^+, or NADH were scanned from 360 to 220 nm.

RESULTS

The results obtained by assaying lactic acid dehydrogenase activity (by initial velocity determinations) in the presence of pesticide are shown in Table I. The compounds possessing an epoxide function (dieldrin, endrin) were about 2 × more inhibitory than chlordane and aldrin; however, the difference in K_i' values is less than half an order of magnitude. Inspection and comparison of the K_i' values in Table I indicate that neither the configuration of the pesticide (endo,exo or endo,endo) nor the presence or absence of an epoxide group is the major inhibitory factor. For this reason, only one compound was chosen for further assay with respect to the coenzyme. Figure 1 shows the determination of K_i' and type of inhibition with respect to both pyruvate and NADH for dieldrin. For both substrates, K_i' is 0.03 mM and inhibition is competitive.

After 1 week at room temperature, the solutions upon inspection for visible studies showed no color development; no new peaks or shifts were observed through scanning (340–800 nm). In the ultraviolet studies, mixtures of adenine–dieldrin and nicotinamide–dieldrin showed no change in the spectral profiles of the nucleosides. However, with NAD^+–dieldrin or NADH–dieldrin, enhancement of absorption occurred at the characteristic absorption peaks of NAD^+ and NADH. These results are shown in Figure 2.

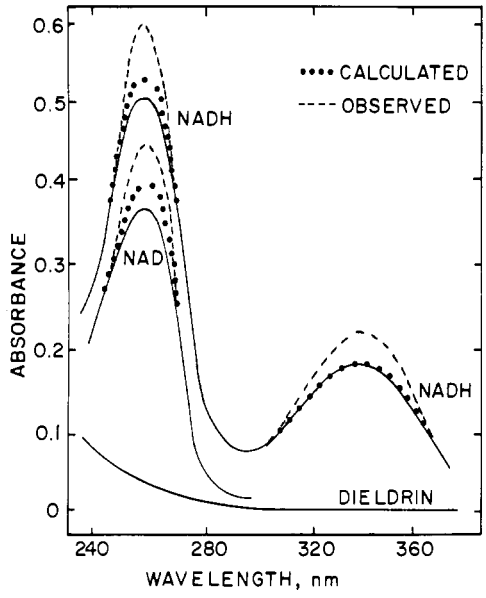


Figure 2. The effect of dieldrin on ultraviolet absorption spectra of NAD^+ and NADH. Experimental conditions: A Cary 14 was used. $[NAD^+] = 0.034$ mM, [NADH] = 0.034 mM, and [dieldrin] = 0.013 mM. Dinucleotides were in 0.1 M phosphate buffer at pH 7.5; pesticides were in buffer and 30% ethanol. Ethanol caused no change in dinucleotide spectra. The solid lines indicate the absorbance of NAD^+ and dieldrin in the presence of ethanol. The expected absorbance due to DDT and NAD(H) was calculated by addition of the separate absorbance of each at 260 and 340 nm.

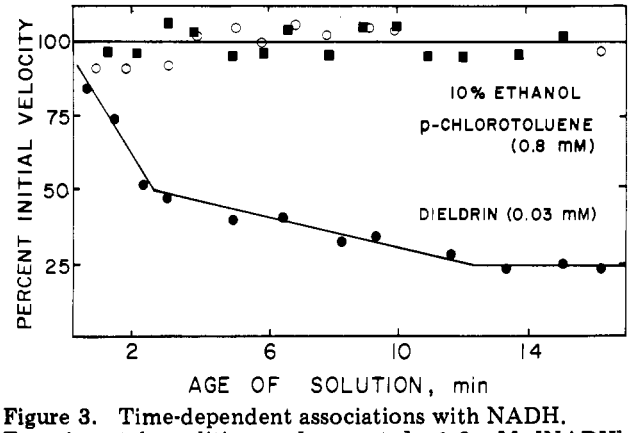


Figure 3. Time-dependent associations with NADH. Experimental conditions: [pyruvate] = 1.3 mM, [NADH] = 0.7 mM, and 1 unit of LDH, in 0.1 M phosphate buffer at pH 7.5, at 25 °C; (•) dieldrin; (○) 10% ethanol; (▪) *p*-chlorotoluene.

For the "time-lag inhibition" studies, reaction mixtures were started with aliquots taken at 1-min intervals from a solution of NADH–buffer, NADH–buffer–ethanol, or NADH–buffer–ethanol–dieldrin. These results are shown in Figure 3. A noninhibitory compound, *p*-chlorotoluene, is included for comparison. Maximum inhibition is not reached for 12 min after the mixing of NADH and dieldrin, indicating a necessity for the occurrence of some type of association between coenzyme and inhibitor.

DISCUSSION

The effect of DDT and Mirex on lactic acid dehydrogenase has been postulated to be a combination of enzyme–inhibitor binding at the active site and association of coenzyme with pesticide, both of which effects yield competitive inhibition (Hendrickson and Bowden, 1973, 1975a,b, 1976a,b) with respect to NADH. Specifically, the enzyme–inhibitor binding is thought to occur at the hydrophobic pocket located at the active site which normally binds the adenosine portion of NADH (Hendrickson and Bowden, 1976b; Einarsson et al., 1974). Competitive inhibition with respect to pyruvate is probably due to interference of the pesticide molecule with pyruvate binding at the second nucleotide site. This theory is supported by the findings of Towell and Woody (1975), who have reported competitive inhibition of lactic acid dehydrogenase with respect to both substrates by bromophenol blue, and have observed using CD methods the binding of this compound to lactic dehydrogenase, apparently at the active site. Bromophenol blue is similar in structure to DDT and other hydrocarbon inhibitors of dehydrogenases known to bind at the adenosine pocket of lactic acid dehydrogenase (Hendrickson and Bowden, 1976b; Einarsson et al., 1974).

The postulated association of NADH and pesticide is not of the charge-transfer type, since no new peak or even a major shift is observed, either in the visible or ultraviolet range and is for that reason difficult to characterize. Ultraviolet studies revealed no change at all in the spectra of adenine or nicotinamide in the presence of dieldrin, indicating that these two portions of the NADH molecule do not separately associate with the dieldrin molecule. Although no shift occurred, both NAD^+ and NADH in the presence of dieldrin underwent an enhancement of absorption, about 10%, at characteristic absorption peaks (340 and 260 nm). This phenomenon is an indication that weak association of coenzyme and inhibitor occurs (Kosower, 1965). In addition, the time-lag inhibition studies shown in Figure 3 indicate the existence of time-dependent association of some type between NADH and pesticide.

The results of these experiments with dieldrin are very similar to those obtained using Mirex and DDT (Hendrickson and Bowden, 1973, 1975a,b, 1976a,b). We would like to suggest that the mechanism of lactic acid dehydrogenase inhibition in vitro of dieldrin, Mirex, and DDT (representatives of the three major classes of chlorinated hydrocarbon pesticides) is very similar for all three. In addition, taking into consideration the postulated manner of pesticide binding to the adenosine-binding pocket of the enzyme (Hendrickson and Bowden, 1976b) and the similarity of this pocket in various types of enzymes (Blake and Evans, 1974; Ohlsson et al., 1974), it is possible that this mechanism may hold true not only for lactic acid dehydrogenase but for other NAD^+-linked dehydrogenases and other enzymes utilizing adenosine-containing coenzymes (Hendrickson and Bowden, 1976b; Blake and Evans, 1974; Ohlsson et al., 1974).

Further studies in this area are underway in this and other laboratories in an effort to elucidate the as yet unclear (Corbett, 1974) in vivo mechanism of the neuroactive polychlorinated hydrocarbons.

LITERATURE CITED

Blake, C. C. F., Evans, P. R., *J. Mol. Biol.* **84**, 585–601 (1974).
Corbett, J. R., "The Biochemical Mode of Action of Pesticides", Academic Press, New York, N.Y., 1974, pp 165–186.
Dixon, M., *Biochem. J.* **55**, 170–171 (1953).
Edwards, C. A., "CRC Persistent Pesticides in the Environment", Chemical Rubber Co., Cleveland, Ohio, 1970.
Einarsson, R., Eklund, J., Zepperzauer, E., Boiwe, T., Branden, C., *Eur. J. Biochem.* **49**, 41–47 (1974).
Hendrickson, C. M., Bowden, J. A., *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **32**, 235 (1973).
Hendrickson, C. M., Bowden, J. A., *J. Agric. Food Chem.* **23**, 407–409 (1975a).

Hendrickson, C. M., Bowden, J. A., *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **34**, 506 (1975b).
Hendrickson, C. M., Bowden, J. A., *J. Agric. Food Chem.* **24**, 241–244 (1976a).
Hendrickson, C. M., Bowden, J. A., *Biochem. Pharmacol.*, submitted for publication (1976b).
Kosower, E. M., *Prog. Phys. Org. Chem.* **3**, 81–163 (1965).
Lineweaver, H., Burk, D., *J. Am. Chem. Soc.* **56**, 658–666 (1934).
Ohlsson, I., Nordstrom, B., Branden, C. I., *J. Mol. Biol.* **89**, 339–354 (1974).
Stickel, L. F., in "Environmental Pollution by Pesticides", Edwards, C. A., Ed., Plenum, London, 1973.
Towell, J., Woody, R., *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **34**, 545 (1975).

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Metabolism in Human Embryonic Lung Cell Cultures of Three Phenylurea Herbicides: Chlorotoluron, Fluometuron, and Metobromuron

Tsu Hui Lin, Robert E. Menzer,* and Hanson H. North

Three phenylurea herbicides, chlorotoluron [*N*-(3-chloro-4-methylphenyl)-*N'*,*N'*-dimethylurea], fluometuron [*N*-(3-trifluoromethylphenyl)-*N'*,*N'*-dimethylurea], and metobromuron [*N*-(4-bromophenyl)-*N'*-methoxy-*N'*-methylurea], were found to be very resistant to the metabolic action of human embryonic lung (HEL) cells in culture. Over 95% of the recovered radioactivities from these ^{14}C-labeled compounds after incubation with HEL cell cultures remained intact, with less than 3 and 2% of the remainder being organoextractable and water-soluble metabolites, respectively. Oxidative metabolism predominated over hydrolytic metabolism for all three compounds. Oxidative metabolites were identified by cochromatography as follows—for chlorotoluron: *N*-(3-chloro-4-methylphenyl)-*N'*-formyl-*N'*-methylurea, *N*-(3-chloro-4-methylphenyl)-*N'*-formylurea, *N*-(3-chloro-4-methylphenyl)-*N'*-methylurea, and *N*-(3-chloro-4-methylphenyl)urea; for fluometuron: *N*-(3-trifluoromethylphenyl)-*N'*-formyl-*N'*-methylurea, *N*-(3-trifluoromethylphenyl)-*N'*-methylurea, and *N*-(3-trifluoromethylphenyl)urea; and metobromuron: *N*-(4-bromophenyl)-*N'*-methylurea, *N*-(4-bromophenyl)urea, and *N*-(4-bromophenyl)-*N'*-hydroxy-*N'*-methylurea. The amounts of water-soluble metabolites of these compounds were too low for identification.

Cell culture systems provide a very useful method for investigating the direct actions of xenobiotics on cells and tissues in the absence of the complex system in a whole organism (Rosenoer, 1966). Cultured mammalian cells have been used to study metabolism of pesticides. Baron and Locke (1970) reported carbaryl metabolism by a human embryonic lung cell line. North and Menzer (1970) studied growth and esterase inhibition in cultured L-929 mouse fibroblast cells for four organophosphorus insecticides. They also reported biotransformation of dimethoate (North and Menzer, 1972), DDT (North and Menzer, 1973), carbaryl (Lin et al., 1975a), and chlordimeform (Lin et al., 1975b) in primary human embryonic lung cell cultures.

Chlorotoluron, fluometuron, and metobromuron are three phenylurea herbicides. Only a few studies have been reported on the metabolism of these compounds. Mücke et al. (1976) administered [^{14}C]chlorotoluron to rats and found that chlorotoluron was demethylated to *N*-(3-chloro-4-methylphenyl)-*N'*-methylurea and *N*-(3-chloro-4-methylphenyl)urea. It was then further oxidized to form benzylic alcohol derivatives and benzoic acid derivatives. The benzyl alcohol derivative was demethylated to *N*-(3-chloro-4-hydroxymethylphenyl)-*N'*-methylurea. The benzoic acid was subsequently demethylated to *N*-(3-chloro-4-carboxyphenyl)-*N'*-methylurea and *N*-(3-chloro-4-carboxyphenyl)urea. The benzylic alcohol derivatives could also be conjugated. In vitro metabolism of chlorotoluron in rats and Japanese quail has been reported by Hinderer and Menzer (1976a,b).

The metabolism of fluometuron has been investigated mainly in plants. In cotton and cucumber, fluometuron was metabolized to form demethylfluometuron, *N*-(3-trifluoromethylphenyl)urea, and 3-(trifluoromethyl)aniline (Rogers and Funderburg, 1968). The metabolic fate of fluometuron in animals as well as in tissue or cell cultures is still unknown.

The metabolism of metobromuron in plants and animals has been investigated by Geissbühler and Voss (1972). Three possible metabolic pathways were reported: (1) metobromuron undergoes metabolic change to form in sequence, *N*-(4-bromophenyl)-*N'*-methoxyurea, *N*-(4-bromophenyl)-*N'*-hydroxyurea, and *N*-(4-bromophenyl)-urea; (2) metobromuron forms *N*-(4-bromophenyl)-*N'*-hydroxy-*N'*-methylurea, *N*-(4-bromophenyl)-*N'*-hydroxyurea, and *N*-(4-bromophenyl)urea; (3) metobromuron becomes *N*-(4-bromophenyl)-*N'*-hydroxy-*N'*-methylurea, *N*-(4-bromophenyl)-*N'*-methylurea, and *N*-(4-bromophenyl)urea.

The results of investigations on the metabolism of these three phenylurea compounds in primary human embryonic lung cells in culture are reported herein.

MATERIALS AND METHODS

Chemicals. [^{14}C]Chlorotoluron (sp act. 3.77 mCi/mmol) and [^{14}C]fluometuron (sp act. 2.42 mCi/mmol) were labeled on the phenylmethyl groups. [^{14}C]Metobromuron (sp act. 2.1 mCi/mmol) was uniformly labeled on the benzene ring. The radioactive compounds and their unlabeled derivatives were furnished by CIBA-Geigy, Ltd., Basle, Switzerland. Before use each radiolabeled material was purified on TLC using the system appropriate to each

Department of Entomology, University of Maryland, College Park, Maryland 20742.