Screening of Pesticides for Mutagenic Potential using *Salmonella typhimurium* Mutants

Thomas C. Marshall, H. Wyman Dorough,* and H. Earle Swim

The mutagenic activity of several pesticides and related analogues was examined with a set of four strains of *Salmonella typhimurium* (Ames assay, TA1535 series; deep rough strains without excision repair). Nitrosocarbaryl, a derivative of the insecticide carbaryl, proved to be a potent base-pair substitution mutagen showing activity at 0.5 μg/plate, as well as demonstrating a relatively mild frameshift activity. Captan at 25 μg/plate showed both frameshift and base-pair substitution mutagenesis. The mutagenic properties of these two compounds decreased when exposed to rat liver homogenate. DDT, DDE, heptachlor, heptachlor epoxide, dieldrin, carbaryl, linuron, and diazinon were not mutagenic in this system.

A wide variety of test systems with differing degrees of simplicity and sensitivity have been developed to detect the mutagenic and carcinogenic potential of chemicals, tobacco smoke fractions, and extracts of airborne pollutants. The methods involve use of cultured cells, microorganisms, plants, or intact animals, and have been recently reviewed by Stolz et al. (1974) and Epstein and Legator (1971).

Ames et al. (1973a,b) have introducted an assay for the detection of mutagens using histidine-requiring mutants of *Salmonella typhimurium*. The assay is quite sensitive to detect point mutations of both the frameshift and base-pair substitution type, is easy to perform, and includes a capacity to approximate mammalian metabolism by the addition of a rat liver homogenate fraction. In the present study, we have employed the Ames assay procedure to evaluate the mutagenic potential of several pesticides which have been used extensively for the control of crop and animal pests.

MATERIALS AND METHODS

Chemicals. Nitrosocarbaryl (*N*-nitroso-*N*-methyl-1-naphthylcarbamate) was prepared according to procedures outlined by Elespuru et al. (1974). The structure was confirmed by a mass spectral analysis (70 eV), which showed a parent ion at *m/e* 230 and fragments at *m/e* 143 (naphthoxyl), *m/e* 127 (naphthyl), and *m/e* 115, as previously reported. Purity was confirmed by silica gel thin-layer chromatography in three solvent systems: 9:1 carbon tetrachloride–ether; 1:1 chloroform–ether; and 1:1 ethyl acetate–ether. DDT, DDE, dieldrin, heptachlor, heptachlor epoxide, carbaryl, diazinon, linuron, and captan were obtained in a pure form from stocks of standards used in residue analysis. All the compounds were dissolved in dimethyl sulfoxide in concentrations varying from 1 to 10 mg/ml. Captan, a fungicide, was included primarily because of its known mutagenic activity (Kada et al., 1974; McCann et al., 1975). Nitrosocarbaryl is a derivative of the insecticide carbaryl which theoretically could be formed in the stomach if residues of carbaryl and nitrites in the form of food additives were present simultaneously (Elespuru et al., 1974). The other compounds are common pesticides with the exception of DDE and heptachlor epoxide which are metabolites of DDT and heptachlor, respectively.

Bacterial System. The bacterial strains (TA1535, TA1536, TA1537, and TA1538) were acquired from Dr. Ames (Biochemistry Department, University of California, Berkeley). These are histidine-requiring mutants which, in addition, lack an excision repair system and the lipopolysacchride barrier making them more sensitive and permeable to foreign compounds. Strain TA1535 (Ames et al., 1973b) is a base-pair substitution mutant while the other three are frameshift mutants.

The agar medium was prepared according to Ames et al. (1973b) as was the "S-9" liver preparation. Induction of the liver enzyme system was accomplished by providing 200 g Sprague-Dawley male rats with drinking water containing 0.1% phenobarbital for 7 days. This treatment greatly increases the mixed function oxidase activity which is responsible for most drug metabolism.

The test methods used were exactly as described by Ames et al. (1973a). Up to 0.5 ml of Me_2SO solution was added with the soft agar to each plate as was 0.1 ml of bacterial tester strain culture (10^9 bacteria/ml estimated by measuring turbidity on a Klett spectrophotometer). Those plates assayed with the rat liver homogenate contained 0.5 ml of the "S-9" mixture. The plates were incubated for 48 h at 37 °C and data quantitated by counting the number of colonies per plate, each of which represents mutation from the histidine-dependent to the histidine-independent form. 2-Acetylaminofluorene and *N*-methyl-*N*-nitro-*N'*-nitrosoguanidine were used as active standards or positive controls in the prescribed manner (Ames et al., 1973a). Cellular toxicity was determined by mixing each compound with the tester strain as described above, but with the top agar containing an excess of histidine (3 μmol/plate). These were incubated for 24 h at 37 °C and evaluated for growth inhibition by comparison to control plates.

RESULTS AND DISCUSSION

The variability of the spontaneous reversion frequencies in the four tester strains in all series of test is illustrated in Table I. The considerable variation observed could have been narrowed by two procedures: (1) expressing the reversion frequency in terms of number per million viable bacteria, which would require a dilution series plate count on each date of testing; and (2) standardizing the stock cultures each time they are grown (every 7 days) to 10^9 bacteria per ml by a dilution series plate count instead of estimating this on a spectrophotometer. However, these

Department of Entomology, University of Kentucky, Lexington, Kentucky 40506 [Department of Entomology and Graduate Program in Toxicology, University of Kentucky, Lexington, Kentucky 40506 (T.C.M., H.W.D.) and the Department of Cell Biology, Medical Science Center, Lexington, Kentucky 40506 (H.E.S.)].

Table I. Spontaneous Reversion Frequency in TA1535 Series of *S. typhimurium* with (+) and without (−) Rat Liver Homogenate

Parameters	Spontaneous reversion per 10^8 bacteria/strain							
	1535		1536		1537		1538	
	−	+	−	+	−	+	−	+
Mean	10.7	10.3	0.6	2.7	10.2	15.0	17.3	32.1
Standard deviation	±7.0	±5.7	±1.1	±3.5	±4.0	±8.6	±6.7	±11.1
Range	1-35	1-28	0-5	0-15	1-22	2-43	1-43	15-56
No. of samples	49	38	33	28	49	43	59	49

Table II. Mutagenicity in *S. typhimurium*, TA1535 Series

Compd	μg/plate, ±RLH[a]	Revertant colonies per plate/strain				Toxic[b] level, μg ±RLH
		1535	1536	1537	1538	
DDT	2500−	6 (8)	0 (0)	10 (15)	22 (19)	>2500−
	2500+	15 (17)	3 (8)	45 (43)	69 (56)	>2500+
DDE	1000−	4 (7)	0 (1)	10 (13)	17 (13)	>2500−
	1000+	18 (17)	3 (8)	22 (43)	69 (56)	>1000+
Dieldrin	1000−	3 (8)	0 (1)	8 (11)	16 (18)	>2500−
	1000+	17 (17)	18 (8)	37 (43)	63 (56)	>1000+
Heptachlor	1000−	4 (10)	1 (0)	16 (11)	12 (12)	>2500−
	1000+	8 (17)	8 (8)	41 (43)	53 (56)	>1000+
Heptachlor epoxide	1000	10 (13)	0 (0)	10 (13)	14 (10)	>2500−
	1000+	15 (17)	7 (8)	55 (43)	65 (56)	>1000+
Diazinon	1000−	12 (10)	1 (0)	9 (11)	13 (13)	>1000−
	1000+	22 (17)	7 (2)	3 (18)	13 (33)	>1000+
Carbaryl	1000−	19 (9)	0 (2)	19 (11)	22 (23)	>2500−
	1000+	9 (17)	10 (8)	33 (43)	51 (56)	>1000+
Nitrosocarbaryl	0.5−	51 (8)				100−
	50+	870 (17)	2 (2)	21 (8)	56 (56)	>1000+
	50−	2932 (8)	0 (0)	92 (15)	96 (20)	100−
Linuron	25−	8 (8)	2 (0)	4 (22)	13 (28)	50−
	200+	11 (13)	1 (1)	8 (17)	40 (46)	~400+
Captan	10−	248 (10)	0 (1)	335 (7)	19 (32)	100−
	25−	320 (10)	2 (1)	47 (7)	11 (32)	
	25+	117 (14)	2 (1)	148 (9)	32 (32)	~400+
	50−	596 (10)	0 (1)	0 (7)	0 (32)	
	50+	231 (14)	0 (1)	288 (9)	51 (32)	

[a] With (+) and without (−) rat liver homogenate (RLH) fraction. [b] Values preceded by > were the highest level tested. Inhibition of bacterial growth was used as an indication of toxicity. Numbers enclosed by parentheses are the spontaneous reversion rates for each test.

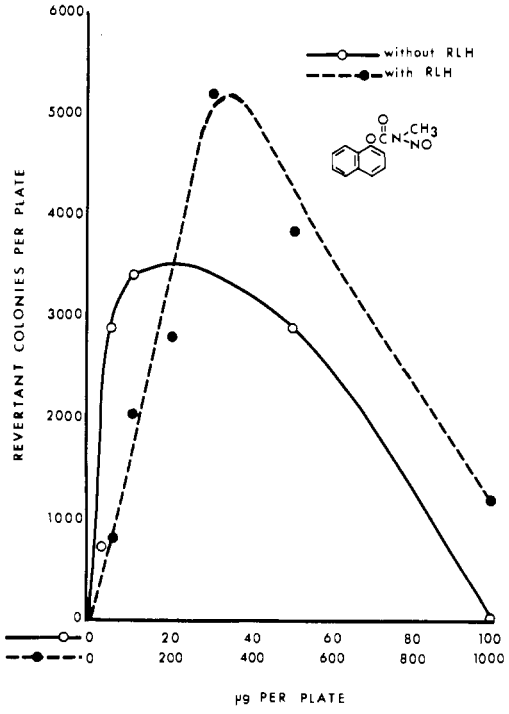

Figure 1. Mutagenicity of nitrosocarbaryl to *Salmonella typhimurium* (strain TA1535) with and without rat liver homogenate (RLH).

operations were considered unnecessary for the purpose of screening large numbers of chemicals for mutagenic potential.

Table II summarizes the mutagenic activity of the ten compounds tested. The control values represent the spontaneous reversion frequencies of the tester strains both with and without rat liver homogenate. Where no activity was observed, only the highest dose tested or the maximum tolerated concentration (the highest concentration which did not appreciably inhibit growth) is listed. However, all the compounds were evaluated at concentrations as low as 50 μg/plate with each successive level increased by a factor of two up to the highest level. The toxicity data were useful in determining the concentrations that gave maximum reversion with a minimum of growth inhibition, an influence that would obviously affect the dose–response correlation. As can be seen in Figure 1, the reversion frequency with nitrosocarbaryl decreased at higher concentrations, presumably due to toxicity resulting from multiple mutations which tend to inactivate essential genes on the chromosome (Ames et al., 1973a).

The results revealed two active compounds, captan and nitrosocarbaryl. Both are active as base-pair substitution mutagens as evidenced by their ability to back mutate the 1535 strain. According to Ames et al. (1973b), the exact base-pair changes which revert this strain are not clear, but it is suspected that most, if not all, of the six possible base-pair substitutions can be detected.

Nitrosocarbaryl was extremely potent, demonstrating increased reversion at the 0.5-μg level in the base substitution revertible strain 1535. This compound has demonstrated mutagenic characteristics in two other bacteria, *Escherichia coli* and *Haemophilus influenzae* (Elespuru et al., 1974). Beattie and Kimball (1974) reported that mutagenesis induced in *H. influenzae* by nitrosocarbaryl consists of both replication-dependent and replication-independent components. Our results with strains 1537 and 1538 indicate that nitrosocarbaryl may be slightly active as a frameshift mutagen at higher concentrations. TA1538 is particularly sensitive to the polycyclic aromatic hydrocarbons due to a specific "hot spot" in the DNA (Ames et al., 1973b; Yourno, 1972). Presence of the "S-9" liver homogenate decreased the mutagenic action of nitrosocarbaryl, thus indicating that activation was not required.

Four series of tests on nitrosocarbaryl in TA1535 representing four different stock cultures of that strain varying in age from 2 to 7 days were combined for a regression analysis. It was found that the age of the culture had no significant contribution to the variability of the system. Utilizing all the data generated in this study with nitrosocarbaryl, a correlated t test showed that the 1-μg level of nitrosocarbaryl without rat liver homogenate and the 50-μg level with rat liver homogenate were the concentration levels showing the least significant difference ($P < 0.01$) from the spontaneous reversion frequency.

Based on the results with nitrosocarbaryl and assuming that the variabilty is inherent within the method regardless of the activity of the compound, a hypothetical compound tested twice would need to demonstrate a reversion mean at the peak response level of at least 135 without rat liver homogenate, and at least 320 with rat liver homogenate in order to conclude that the compound was mutagenic in strain 1535 ($P = 0.05$). This conservative approach is necessary to eliminate the false positives which could occur if one attempted to apply the standard t test. However, the strict adherence to numbers obtained from such a statistical analysis is not recommended until a large number of active compounds are studied under similar conditions. Even then the results should be viewed only as a guideline by the researcher.

Captan was much less potent than nitrosocarbaryl, but also was active as a frameshift mutagen in strain 1537. Similarly, it seemed to act directly since the liver homogenate decreased its activity. However, activation via the metabolic pathways of the bacteria cannot be ruled out completely for captan or nitrosocarbaryl. Several in vitro studies (Epstein and Shafner, 1968; Legator et al., 1969) have shown captan to be mutagenic, while results of other in vivo studies fail to correlate with these findings (Kennedy et al., 1975). This may be due to captan's short half-life in serum (Epstein and Legator, 1971).

The other eight compounds were negative in this system. Unless toxicity was noted, they were tested in concentrations up to 2500 μg/plate. Ideally, compounds should be tested up to levels of growth inhibition as was done with linuron, but at the highest levels shown in Table II, precipitation was seen with the chlorinated hydrocarbons and with carbaryl. Each of the eight compounds found inactive in the current study has demonstrated varying degrees of morphological changes in chromosomes or effects on meiosis and mitosis, mostly in plant assay systems (Vaarama, 1947; Amer, 1965; Markaryan, 1967; Wuu and Grant, 1967; Epstein and Shafner, 1968; Kaszubiak, 1968; Legator et al., 1969). Moreover, the use of DDT, dieldrin, and heptachlor has been banned or severely limited by the Environmental Protection Agency because of their carcinogenic activity in experimental mammals. A lack of activity of these chemicals in the Ames assay may be indicative of its insensitivity to certain types of plant and animal mutagens–carcinogens, or of the inconclusiveness of studies conducted in higher organisms.

Given the chemical structures and the theory on alkylating agents pertaining to their mechanism of mutagenesis, the results obtained in these experiments are somewhat predictable. Both nitrosocarbaryl and captan have good "leaving groups"; thus they could be expected to interact with cellular nucleophiles such as DNA and proteins. Conversely, the other compounds have no such easily identifiable group. Of course, this does not rule out their being active by other mechanisms, as their activity in the test systems noted in the preceding paragraph would suggest.

As part of the report prepared for the Mrak Commision (Secretary's Commission on Pesticides and Their Relationship to Environmental Health) in 1970, Epstein and Legator (1971) recommended a program for mutagenesis testing of pesticides. This included three mammalian test systems (the dominant lethal, host mediated, and in vivo cytogenetic), and several ancillary microbial systems for detecting both single nucleotide changes and effects involving more than one gene. Results reported herein indicate that the Ames assay would be a valuable asset to such a battery of tests, because of its sensitivity, ease of performance, and mammalian metabolizing capacity. Not only would the assay aid in evaluating the mutagenic potential of a pesticide, but also its carcinogenic hazard, since a very high correlation exists among known carcinogens and their ability to back mutate the TA1535 series of *S. typhimurium* (Ames et al., 1973a).

As pointed out by Kennedy et al. (1975), caution should be used in extrapolating effects observed in bacterial strains to higher organisms, since they found no evidence that captan is a genetic hazard in mammalian species even though it has the ability to produce mutations in bacteria. Certainly there exists no single routine procedure which will uncover all the potential hazards of captan or any other chemical. Nonetheless, the damage that may ensue upon this and future generations by chemical mutagenesis and carcinogenesis necessitates that all available resources and techniques to prevent such disastrous consequences are investigated. Of equal importance is the need to continually scrutinize the evaluation procedures so that no product of potentially great social benefit is unnecessarily cast aside.

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Hexachlorobenzene Contamination in Laboratory Monkey Chow

Raymond S. H. Yang,* W. F. Mueller,[1] H. K. Grace, L. Golberg, and F. Coulston

The presence of hexachlorobenzene contamination in some samples of Purina monkey chow was established on the basis of gas chromatographic and mass spectrometric evidence. Among some 30 batches of Purina monkey chow analyzed, the level of hexachlorobenzene ranged from less than 1 ppb to 21.1 ppm. Traces of hexachlorobenzene, estimated to be less than 1 ppb, were also present in several batches of Wayne monkey chow.

Hexachlorobenzene (HCB), a fungicide and a chemical intermediate in industrial organic synthesis, has, in recent years, caused increasing concern as an environmental contaminant. It was first recognized as a hazardous chemical in the early sixties after an episode of massive human poisoning (porphyria cutanea tarda) in Turkey, resulted from the consumption of bread prepared from wheat contaminated with HCB (Schmid, 1960; Cam and Nigogosyan, 1963). Soon thereafter, HCB residues were found in the tissues of various species of wildlife, of domestic animals and humans, and in various food sources (Vos et al., 1968; Koeman et al., 1969; Acker and Schulte, 1970a,b; Tuinstra, 1971; Zeman et al., 1971; Brady and Siyali, 1972; Gilbertson and Reynolds, 1972; Goursand et al., 1972; Newton and Greene, 1972; Smith, 1972; Dejonckheere et al., 1974; Johnson et al., 1974). In a recent report (National Academy of Sciences, 1975) HCB was singled out as the only organic chemical contaminant present in the ocean at levels likely to cause serious problems.

During the course of a comparative metabolic study of hexachlorobenzene (HCB) in rhesus monkeys and rats (Yang and Pittman, 1975), fecal samples from untreated control monkeys were used for quantitative recovery of added HCB by gas chromatography. Significant quantities of HCB were detected consistently in the control monkey feces. Since every piece of glassware, other apparatus, and various reagents had been examined routinely by gas chromatography before each experiment, the source of HCB found was definitely the fecal material, from which the diet was incriminated.

This paper reports the evidence of HCB contamination in Purina monkey chow. The methods of sampling, extraction, clean-up, chromatographic, and mass spectrometric analysis of HCB from monkey feces and monkey chows are described.

Institute of Comparative and Human Toxicology, Albany Medical College, Albany, New York 12208.
[1]Present address: Institut für Oekologische Chemie, Gesellschaft für Strahlen-und Umweltforschung mbH, Munich, Germany.

EXPERIMENTAL SECTION

The various batches of Purina monkey chow (micromixed) used in this study were purchased from Hoosac Valley Feed and Grain Co., North Adams, Mass. On each bag (25 lb/bag) of the Monkey chow, there was a white information tag bearing the number 5038. The sampling and analysis of the Purina monkey chow were conducted in two different periods of time in the following manner.

(1) Over an 8-month period between October 1974 and May 1975, random sampling of 19 batches (200 g/batch) of monkey chow was carried out from freshly opened bags in our animal facility. Since the rate of turnover of monkey chow in our animal facility was approximately 3 bags/day, this sampling was therefore made from an estimated quantity of 18000 lb of monkey chow.

(2) In June 1975, five bags of Purina monkey chow were purchased directly from the same distributor. Upon consultation with the Ralston Purina Company, the following identification numbers were noted on the tape closure on the bottom of the bags: 0122751BT on three of the bags, 1009742 and 0102752 on the remaining two bags, respectively. The first seven digits denote, in sequence, the month, day, year, and the shift of the production. Thus, the number of 1009742 indicated that the product was manufactured on October 9, 1974 on the second shift. Two to three analyses were made on the monkey chow from each of these five bags.

Four batches of Wayne monkey diet were also analyzed during this study. They were purchased from R & E Feed Co., Troy, N.Y., and the bags carried an information tag bearing the number 8663-00.

Monkey feces were collected and pooled from various stock monkeys (*Macaca mulatta*) or from control monkeys on other experiments. These monkeys had no prior history of exposure to HCB and were not known to be in contact with pesticide in their well-ventilated holding rooms. At