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Evaluation of Herbicides for Possible Mutagenic Properties

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One-hundred-and-ten herbicides were evaluated for their ability to induce point mutations in one or more of four different microbial systems. None of the herbicides appeared to cause point mutations in these microbial systems in comparison with known mutagens such as 5-bromouracil or 2-aminopurine. Except for inconclusive evidence relating to four herbicides within one test of one system, mutagenic rates of herbicide-treated organisms did not differ significantly from spontaneous rates. In this one test, four herbicides were associated with mutation frequencies slightly in excess of the control. The observed increases were small, and the rates of mutation were lower than spontaneous rates of controls in other tests of the same system. Therefore, it appears that the increases observed with these four herbicides were within the normal range of spontaneous rates.

Pesticide chemicals are used widely to control insects, nematodes, plant diseases, and weeds. These chemicals and their residues enter the biological food chain at various points and for varying periods. Some of these compounds may be toxic to desirable organisms such as crops, wildlife, and man. It is uncertain whether some may also be mutagenic. Consequently, they might cause alterations of the genetic material of living cells as a result of chronic exposures below the toxic level. A number of reports are available concerning nuclear (or chromosomal) aberrations in plants, which suggests that herbicides should be evaluated for possible mutagenicity (Northrop, 1963; Suneson and Jones, 1960; Suneson *et al.*, 1965; Wuu and Grant, 1966, 1967).

Accordingly, we have undertaken and completed an examination of more than 100 herbicides and other chemicals for their ability to cause point mutations in certain microbiological systems. In this study we have used as evaluation systems eight histidine-requiring mutants of *Salmonella typhimurium*, bacteriophage T_4, and two rII mutants of bacteriophage T_4. These test systems have been reported (Crow, 1968) to provide a high probability of detecting genetic damage of the point mutation type, to single genophores, or to the DNA molecule. These tests have been generally recommended as a first step on the evaluation of chemicals for mutagenic properties and have the advantage of being a qualitative and quantitative assessment of a large number of compounds. These tests are designed to detect mutagens causing base substitutions, deletions or additions, or grosser alterations, but do not detect mutations involving DNA transformations or genetic alterations caused by chromosome breaks or other chromosome changes which are restricted to diploid cells.

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EXPERIMENTAL

Bacterial Strains and Viruses. Eight histidine-requiring mutants of *Salmonella typhimurium* were obtained from Bruce N. Ames of the University of California, Berkeley. These mutants involve the *C*, *D*, and *G* gene of the histidine operon in *S. typhimurium* and were designated as either nonsense (amber or ochre), missense, or frameshift mutants (Whitfield *et al.*, 1966). The T_4 bacteriophage and *Escherichia coli* B host were supplied by Robert M. McCombs of Baylor University College of Medicine, Houston, Texas. The rII mutant, AP72, and *E. coli* strain KB were obtained from Sewell Champe of Purdue University, Bloomington, Ind., while the rII mutant N17 was obtained from Ernst Freese of the National Institutes of Neurological Diseases and Blindness, NIH, Bethesda. Mutant AP72 is a transition mutant which involves the transition of the guanine–cytosine pair to adenine–thymine pair, while mutant N17 involves the transition from adenine–thymine pair to guanine–cytosine pair.

Cultural Conditions. The mutagenic properties of the test herbicides and chemicals were evaluated with the *Salmonella typhimurium* mutants by measuring the frequency of reversion to histidine independence, using an agar overlay technique according to the procedures described by Ames and Whitfield (1966). The bacteria were exposed to the test compounds on petri plates which were prepared by mixing 0.2 ml of freshly grown cultures (2×10 bacteria per milliliter) of the mutants with 2 ml of 0.6% agar at 45°C. The soft agar, which contained a trace (0.20 μmol) of histidine as well as the bacterial inoculum, was then poured onto plates of histidine-free minimal agar medium. Approximately 1 to 5 μl of liquid

Table I. Response of Histidine-Requiring Mutants of *Salmonella typhimurium* to Known Mutagens and Herbicides

Common name or designation	Chemical name	Test result
	Diethyl sulfate (known mutagen)	+
NG	*N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (known mutagen)	+
ICR-191	Acridine-like compound (known mutagen)	+
Acrolein	Acrolein	−
Ametryne	2-(Ethylamino)-4-(isopropylamino)-6-(methylthio)-*s*-triazine	−
Amiben	3-Amino-2,5-dichlorobenzoic acid	−
Amitrole	3-Amino-*s*-triazole	−
AMS	Ammonium sulfamate	−
Atraton	2-(Ethylamino)-4-(isopropylamino)-6-methyloxy-*s*-triazine	−
Atrazine	2-Chloro-4-(ethylamino)-6-(isopropylamino)-*s*-triazine	−
Barban	4-Chloro-2-butynyl *m*-chlorocarbanilate	−
Benefin	*N*-Butyl-*N*-ethyl-α,α,α-trifluoro-2,6-dinitro-*p*-toluidine	−
Bensulide	*O,O*-Diisopropyl phosphorodithioate *S*-ester with *N*-(2-mercaptoethyl)benzenesulfonamide	−
Bromacil	5-Bromo-3-*sec*-butyl-6-methyluracil	−
Bromoxynil	3,5-Dibromo-4-hydroxybenzonitrile	−
Buturon	3-(*p*-Chlorophenyl)-1-methyl-1-(1-methyl-2-propynyl)-urea	−
Cacodylic acid	Hydroxydimethylarsine oxide	−
CDAA	*N,N*-Diallyl-2-chloroacetamide	−
CDEC	2-Chloroallyl diethyldithiocarbamate	−
Chlorazine	2-Chloro-4,6-bis(ethylamino)-*s*-triazine	−
Chloroxuron	3-[*p*-(*p*-Chlorophenoxy)phenyl]-1,1-dimethylurea	−
Chlorpropham	Isopropyl *m*-chlorocarbanilate	−
CMA	Calcium methanearsonate	−
4-CPA	(4-Chlorophenoxy)acetic acid	−
CPMF	1-Chloro-*N*-(3,4-dichlorophenyl)-*N,N*-dimethylformamidine	−
4-CPP	2-(4-Chlorophenoxy)propionic acid	−
Cycluron	3-Cyclooctyl-1,1-dimethylurea	−
Cypromid	3',4'-Dichlorocyclopropanecarboxanilide	−
2,4-D	(2,4-Dichlorophenoxy)acetic acid	−
3,4-DA	(3,4-Dichlorophenoxy)acetic acid	−
Dalapon	2,2-dichloropropionic acid	−
Dazomet	Tetrahydro-3,5-dimethyl-2*H*-1,3,5-thiadiazine-2-thione	−
2,4-DB	4-(2,4-Dichlorophenoxy)butyric acid	−
3,4-DB	4-(3,4-Dichlorophenoxy)butyric acid	−
DCB	*o*-Dichlorobenzene	−
DCPA	Dimethyl tetrachloroterephthalate	−
2,4-DEP	Tris[2-(2,4-dichlorophenoxy)ethyl] phosphite	−
Desmetryne	2-(Isopropylamino)-4-(methylamino)-6-(methylthio)-*s*-triazine	−
Diallate	*S*-(2,3-Dichloroallyl) diisopropylthiocarbamate	−
Dicamba	3,6-Dichloro-*o*-anisic acid	−
Dichlobenil	2,6-Dichlorobenzonitrile	−
Dichlone	2,3-Dichloro-1,4-naphthoquinone	−
Dichlorprop	2-(2,4-Dichlorophenoxy)propionic acid	−
Dicryl	3',4'-Dichloro-2-methylacrylanilide	−
Dinoseb	2-*sec*-Butyl-4,6-dinitrophenol	−
Diphenamid	*N,N*-Dimethyl-2,2-diphenylacetamide	−
Diphenatrile	Diphenylacetonitrile	−
Dipropalin	*N,N*-Dipropyl-2,6-dinitro-*p*-toluidine	−
Diquat	6,7-Dihydrodipyrido[1,2-*a*:2',1'-*c*]pyrazinediium salt	−
Diuron	3-(3,4-Dichlorophenyl)-1,1-dimethylurea	−
DMPA	*O*-(2,4-Dichlorophenyl) *O*-methyl isopropylphosphoramidothioate	−
DNOC	4,6-Dinitro-*o*-cresol	−
EBEP	Ethyl bis-(2-ethylhexyl)phosphinate	−
Endothall	7-Oxabicyclo[2.2.1]heptane-2,3-dicarboxylic acid	−
EPTC	*S*-Ethyl dipropylthiocarbamate	−
Erbon	2-(2,4,5-Trichlorophenoxy)ethyl 2,2-dichloropropionate	−
Fenac	(2,3,6-Trichlorophenyl)acetic acid	−
Fenuron	1,1-Dimethyl-3-phenylurea	−
Fenuron TCA	1,1-Dimethyl-3-phenylurea monotrichloroacetate	−
Ioxynil	4-Hydroxy-3,5-diidobenzonitrile	−
Ipatone	2-(Diethylamino)-4-(isopropylamino)-6-methoxy-*s*-triazine	−
Ipazine	2-Chloro-4-(diethylamino)-6-isopropylamino)-*s*-triazine	−
Isocil	5-Bromo-3-isopropyl-6-methyluracil	−
KOCN	Potassium cyanate	−
Lenacil	3-Cyclohexyl-6,7-dihydro-1*H*-cyclopentapyrimidine-2,4(3*H*,5*H*) dione	−
Linuron	3-(3,4-Dichlorophenyl)-1-methoxy-1-methylurea	−
MAA	Methanearsonic acid	−
MCPB	4-[(4-Chloro-*o*-tolyl)oxy]butyric acid	−
Mecoprop	2-[(4-Chloro-*o*-tolyl)oxy]propionic acid	−
Metham	Sodium methyldithiocarbamate	−
MH	1,2-Dihydro-3,6-pyridazinedione	−
Molinate	*S*-Ethyl hexahydro-1*H*-azepine-1-carbothioate	−
Monolinuron	3-(*p*-Chlorophenyl)-1-methoxy-1-methylurea	−
Monuron	3-(*p*-Chlorophenyl)-1,1-dimethylurea	−

Table I. (*Continued*)

Common name or designation	Chemical name	Test result
Monuron TCA	3-(*p*-Chlorophenyl)-1,1-dimethylurea mono(trichloroacetate)	–
MSMA	Monosodium methanearsonate	–
Naptalam	*N*-1-Naphthylphthalamic acid	–
Neburon	1-Butyl-3-(3,4-dichlorophenyl)-1-methylurea	–
Norea	3-(Hexahydro-4,7-methanoindan-5-yl)-1,1-dimethylurea	–
Paraquat	1,1′-Dimethyl-4,4′-bipyridinium salts	–
PBA	Chlorinated benzoic acid	–
PCP	Pentachlorophenol	–
Pebulate	*S*-Propyl butylethylthiocarbamate	–
Picloram	4-Amino-3,5,6-trichloropicolinic acid	–
PMA	(Acetato)phenylmercury	–
Prometone	2,4-Bis(isopropylamino)-6-methoxy-*s*-triazine	–
Prometryne	2,4-Bis(isopropylamino)-6-(methylthio)-*s*-triazine	–
Propanil	3′,4′-Dichloropropionanilide	–
Propazine	2-Chloro-4,6-bis(isopropylamino)-*s*-triazine	–
Propham	Isopropyl carbanilate	–
Pyrazon	5-Amino-4-chloro-2-phenyl-3(2*H*)-pyridazinone	–
Pyriclor	2,3,5-Trichloro-4-pyridinol	–
Sesone	2-(2,4-Dichlorophenoxy)ethyl sodium sulfate	–
Siduron	1-(2-Methylcyclohexyl)-3-phenylurea	–
Silvex	2-(2,4,5-Trichlorophenoxy)propionic acid	–
Simazine	2-Chloro-4,6-bis(ethylamino)-*s*-triazine	–
Simeton	2,4-Bis(ethylamino)-6-methoxy-*s*-triazine	–
Simetryne	2,4-Bis(ethylamino)-6-(methylthio)-*s*-triazine	–
Solan	3′-Chloro-2-methyl-*p*-valerotoluidide	–
Swep	Methyl 3,4-dichlorocarbanilate	–
2,4,5-T	(2,4,5-Trichlorophenoxy)acetic acid	–
2,4,5-TB	4-(2,4,5-Trichlorophenoxy)butyric acid	–
2,3,6-TBA	2,3,6-Trichlorobenzoic acid	–
TCA	Trichloroacetic acid	–
TCBA	Trichlorobenzene	–
Terbacil	3-*tert*-Butyl-5-chloro-6-methyluracil	–
2,2,3-TPA	2,2,3-Trichloropropionic acid	–
Triallate	*S*-(2,2,3-Trichloroallyl) diisopropylthiocarbamate	–
Tricamba	3,5,6-Trichloro-*o*-anisic acid	–
Trietazine	2-Chloro-4-(diethylamino)-6-(ethylamino)-*s*-triazine	–
Trifluralin	α,α,α-Trifluoro-2,6-dinitro-*N*,*N*-dipropyl-*p*-toluidine	–
Trimeturon	1-(*p*-Chlorophenyl)-2,3,3-trimethylpseudourea	–
Vernolate	*S*-Propyl dipropylthiocarbamate	–

test chemicals or small crystals of solid test chemicals were applied directly to the surface of each plate after the top layer of agar, containing the mutant bacteria, had solidified.

The rates of spontaneous reversion of the eight mutants to a form no longer requiring L-histidine for growth were also determined and found to be low, ranging from 0 to 20 colonies per plate.

In addition to the *Salmonella* test system, T_4 bacteriophage was used to evaluate herbicides for mutagenic properties. This phase of the study involved the use of T_4 bacteriophage to detect chemically induced mutations of the rII type (Benzer, 1955) which might be caused by herbicides. The procedure followed was that of Benzer and Freese (1958), who reported on the induction of rII mutants of T_4 bacteriophage by 5-bromouracil (5-BU). A culture of *E. coli* B was prepared by inoculating 20.0 ml of a sulfanilamide-containing medium and aerating for 3.5 hr at 37°C (approximately 2.0×10^8 cells/ml). After 3.5 hr incubation, the herbicide to be evaluated was added in the appropriate concentration and 4×10^4 T_4 phage particles were added simultaneously. A 5-min period was allowed for adsorption of phage to bacteria and then one drop of the mixture was rapidly distributed into test tubes and incubated at 37°C for 30 min. Twenty tubes were used for each herbicide while 40 tubes were used for 5-BU and 40 tubes were used to determine the rate of spontaneous mutation. The number of mutant plaques, which characteristically were larger, with a clear center and a sharp edge, was compared to the normal T_4 plaques which were small, with a clear center surrounded by a halo. Those herbicides not completely soluble in water were first dissolved in a small amount of acetone (0.5 to 1.0 ml) and then brought up to volume with distilled water. Selected herbicides from all of the major classes were evaluated.

Experiments were also performed using two rII mutants of T_4 bacteriophage, designated as AP72 and N17, to detect reversions from the mutant to the wild type which might be caused by the herbicides. The mutant AP72 is a 2-aminopurine-induced rII mutant while N17 is 5-bromouracil-induced mutant. The procedure followed was that of Freese (1959a) with the incorporation of several modifications described by McGahen and Hoffmann (1966), who evaluated substituted uracil herbicides for mutagenic properties. *E. coli* B, grown in a glucose–mineral salts medium, was infected with either AP72 or N17 at 37°C at a multiplicity of infection of about 0.1, the phage was allowed to adsorb, and the infected cells were diluted in a supplemented mineral salts medium containing 10 μg/ml each of uracil, adenine sulfate, and guanine sulfate, plus 1 μg/ml of 5-fluorodeoxyuridine (supplied by W. E. Scott, Hoffmann-La Roche, Inc., Nutley, N. J.) used to block thymine synthesis. The controls contained 20 μg/ml of thymine. Incubation was carried out for 60 min at 37°C, the time and temperature being chosen as optimal for phage production under our conditions. Chloroform was added and the cultures were shaken for 20 min. The resulting phage

Table II. List of Commercially Formulated Herbicides Evaluated for Possible Mutagenic Properties in the Presence of Histidine-Requiring Mutants of *Salmonella typhimurium*[a]

Common name or designation	Chemical name
Amitrole	3-Amino-(*s*)-triazole (50%)
Bromacil	5-Bromo-3-*sec*-butyl-6-methyluracil (50%)
CDAA	*N*,*N*-Diallyl-2-chloroacetamide (47%)
Atrazine	2-Chloro-4-(ethylamino)-6-(isopropylamino)-*s*-triazine (80%)
Linuron	3-(3,4-Dichlorophenyl)-1-methoxy-1-methylurea (50%)
Dalapon	2,2-Dichloropropionic acid, sodium salt (85%)
2,4-D and dicamba	Diethanolamine salt of (2,4-dichlorophenoxy)-acetic acid (20.1%) and diethanolamine salt of dicamba (1.9%)
Diquat	6,7-Dihydrodipyrido[1,2-*a*:2′,1′-*c*]pyrazinediium ion (35.3%)
2,4-D and dicamba	Dimethylamine salt of (2,4-dichlorophenoxy)-acetic acid (12%) and dimethylamine salt of dicamba (2.4%)
DCPA	Dimethyl ester of tetrachloroterephthalic acid (2.5%)
Diphenamid	*N*,*N*-Dimethyl-2,2-diphenylacetamide (50%)
EPTC and 2,4-D	*S*-Ethyl dipropylthiocarbamate (46.9%) isooctyl ester of (2,4-dichlorophenoxy)acetic acid (35.4%)
2,4,5-T and 2,4-D	(2,4,5-Trichlorophenoxy)acetic acid, butoxyethanol ester (11%) and (2,4-dichlorophenoxy)acetic acid, butoxyethanol ester (23%)
2,4,-D, 2,4,5-T and silvex	Triethanolamine (2,4-dichlorophenoxy)acetate (15%), triethanolamine (2,4,5-trichlorophenoxy)acetate (5.0%), and triethanolamine [2(2,4,5-trichlorophenoxy)propionate] (3.5%)
Trifluralin	α,α,α-Trifluoro-2,6-dinitro-*N*,*N*-dipropyl-*p*-toluidine (44.5%)

[a] None of these herbicides caused mutation. Known mutagens which did cause mutations in this test were as follows: NG, diethyl sulfate, and ICR-191.

preparations were assayed with *E. coli* B and *E. coli* K on tryptone plates. Since AP72 and N17 form plaques of *E. coli* B but not *E. coli* K, whereas the wild type T_4 and the revertant form plaques on both *E. coli* B and *E. coli* K, the back-mutation from AP72 or N17 to the wild-type can be detected by differential plaque counts. Again, those herbicides not completely soluble in water were first dissolved in acetone as previously described.

In all the bacteriophage experiments standard bacteriophage plating techniques were employed (Adams, 1959).

Diffusion Characteristics of Herbicides Into an Aqueous-Based Nutrient Medium. As part of the evaluation of herbicides with the histidine-requiring mutants of *Salmonella typhimurium*, a study was undertaken using ^{14}C-labeled herbicides to determine and characterize the diffusion of herbicides into an aqueous-based nutrient medium. The four ^{14}C-labeled herbicides used for this experiment were: diuron, atrazine, picloram, and bensulide, with water solubilities of 42, 70, 430, and <50 ppm, respectively.

The procedure followed during these experiments was to apply a small crystal of a solid herbicide or approximately 1 to 5 μl of a liquid herbicide to the surface of uninoculated plates of the agar medium, and incubate at 37°C for 72 hr. After the 72-hr incubation the agar plates were sectioned and the weighed segments added to 10 ml of scintillation-counting fluid [4.0 g of 2,5-diphenyloxazole (PPO) and 100 mg of 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP) per liter of toluene]. The amount of radioactivity present in each sample was determined in a liquid scintillation spectrometer (Packard Model 3375).

Source of Herbicides and Other Chemicals. The following herbicides were purchased from Chem Service, Inc., Media, Pa.: barban, bromacil, cacodylic acid, CDEC, chlorazine, chlorpropham, dalapon, DCB, dicryl, DCPA, dichlone, diphenatrile, diuron, dinoseb, EPTC, fenuron, 4-CPA, propham, monuron, pebulate, PCP, PMA, silvex, 2,4-D and 2,4,5-T. All other herbicides listed in Table I were supplied by their respective manufacturers. These herbicides were all in technical form and were 90 to 99% pure with the exceptions of fenuron·TCA (22%), monuron·TCA (22%), and MSMA (49.6%).

The commercially formulated herbicides (Table II) were purchased from local garden supply stores and The Ohio Farm Bureau Federation, Inc., Columbus, Ohio.

The triazine derivatives, cyanuric acid, 2-amino-4-chloro-6-ethylamino-*s*-triazine, 2-amino-4,6-bis-hydroxy-*s*-triazine, and 2,4-bis-amino-6-hydroxy-*s*-triazine were kindly supplied by Geigy Agricultural Chemicals, Ardsley, N. Y.

The herbicide derivatives, 3-chloroaniline, 4-chloroaniline, 2,4-dichloroaniline, and 3,4-dichloroaniline, were purchased from the Aldrich Chemical Co., while a sample of 3,3′,4,4′-tetrachloroazobenzene was supplied by the Crops Research Division, U.S. Department of Agriculture, Beltsville, Md.

The following compounds all served as known mutagenic chemicals in either the *Salmonella* test system and/or the T_4 bacteriophage system: 2-aminopurine (2-AP), 5-bromouracil (5-BU), ICR-191 (furnished by Institute for Cancer Research, Philadelphia, Pa.), aminophylline, thymine, caffeine, and colchicine (purchased from Sigma Chemical Co.); and *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (NG) and diethyl sulfate were obtained from Aldrich Chemical Co., and Eastman Organic Chemicals, respectively.

RESULTS

Histidine-Requiring Mutants of *Salmonella typhimurium*. All of the herbicides listed in Table I, as well as the known mutagenic compounds diethyl sulfate and NG, were evaluated by all eight mutants of our *S. typhimurium* mutant system. Positive responses were observed with the known mutagens, while all of the herbicides gave negative results, indicating a lack of reversion to a form no longer requiring histidine for growth. Responses were observed with the herbicide CDAA, an acetamide-type herbicide, and dalapon, an aliphatic acid, as zones of inhibition on the plates. These zones of inhibition indicated toxicity to the organism but were not an indication of mutagenicity. Such zones do provide evidence that the herbicide has diffused into the medium. Where zones of inhibition are not observed there is reason to question whether bacterial cells are permeable to herbicides. The extensive literature concerned with microbial degradation of many classes of herbicides (Kearney and Kaufman, 1969) suggests that impermeability should be the exception rather than a common explanation for our negative results. Some colonies also grew out on the plates as the result of the expected spontaneous reversions. Thus the results indicated that the 110 herbicides evaluated were not mutagenic to histidine-requiring mutants of *Salmonella typhimurium*.

Herbicides are seldom applied to soil and plants in the pure form but may be either dissolved or suspended in solvents such as kerosene, xylene, or naphthalene-like compounds. Accordingly, Table II lists 15 commercially formulated herbicides that were also investigated. In the *Salmonella* mutant system, no mutagenic response was observed with any of the commercially formulated compounds. The only observable re-

sponses were zones of inhibition with CDAA and diquat. Certain colony-like areas on the plate exposed to a combination of 2,4,5-T and 2,4-D proved to be a white precipitate formed in a reaction between herbicide and medium.

In addition to evaluating the triazine herbicides listed in Table I, evaluation was extended to include the following triazine derivatives: cyanuric acid, 2-amino-4-chloro-6-ethylamino-*s*-triazine, 2-amino-4,6-bis-hydroxy-*s*-triazine, and 2,4-bis-amino-6-hydroxy-*s*-triazine. These compounds are either known to result or could conceivably result from biological degradation processes. Again, none of these chemicals were observed to give a mutagenic response with any of the *Salmonella* mutants.

Agar Diffusion Studies. Table III illustrates the results of the agar diffusion experiments. The data indicate that the low-solubility test herbicides had sufficient water solubility to diffuse in the agar medium. Of the four chemicals tested, picloram diffused the least rapidly, as judged by transfer of radioactivity; however, radioactivity could be detected all the way out to the edge of the plate. The pattern of results obtained for picloram is indicative of some volatilization of the test compound because similar amounts of activity were found in the 20-, 30-, and 40-mm samples.

Detection of Chemically Induced Mutations in T_4 Bacteriophage. Table IV presents the results of experiments with a known mutagenic agent (5-BU) and 35 herbicides. Representative herbicides from all 12 major classes of herbicides were evaluated. Under the conditions used for mutant

Table III. Diffusion of Radioactively Labeled Herbicides in Vogel–Bonner Agar Medium

Test chemical	Approximate distance from origin, mm	Net counts per gram agar per min
Control (no test chemical)	0	0
	15	0.2
	25	0
	30	0
	40	0
Atrazine	0	20,810
	20	8,817
	30	4,668
	40	2,201
Picloram	0	1,052
	20	629
	30	507
	40	541
Bensulide	0	13,368
	20	3,335
	30	1,803
	40	902
Diuron	0	725,510
	20	18,244
	30	8,132
	40	3,804

Table IV. The Effect of 5-BU and Selected Herbicides on the Induction of rII Mutants of T_4 Bacteriophage

Test chemical	Class of herbicide	Amount of test chemical added	Total no. of plaques	Total no. of rII plaques	Percent mutation frequency
No chemical (spontaneous mutation)		0	8945	10	0.11
5-BU		1000 μg	9974	204	2.04
Amiben	Benzoate	50 μg	1336	2	0.15
Amitrole	Triazole	25 μg	3040	3	0.10
Atraton	Triazine	25 μg	5378	10	0.19
Atrazine	Triazine	20 μg	3957	4	0.10
Bensulide	Sulfonamide	10 μl	3098	8	0.26
Bromacil	Substituted uracil	50 μg	3495	4	0.11
CDAA	Acetamide	10 μl	2046	4	0.20
CDEC	Thiocarbamate	0.05 μl	6442	9	0.14
2,4-D	Phenoxyalkanoate	50 μg	2942	2	0.07
2,4-DEP	Phenoxyalkanoate	10 μl	3761	4	0.11
Dicamba	Benzoate	25 μg	3145	8	0.25
Dalapon	Aliphatic acid	20 μg	5282	7	0.13
Dicryl	Acylanilide	50 μg	2202	1	0.05
DNOC	Substituted phenol	50 μg	3309	4	0.12
Diphenamid	Acetamide	50 μg	2327	2	0.09
Dipropalin	Toluidine	100 μg	2231	6	0.27
Diquat	Pyrazidiinium salt	20 μg	7662	9	0.12
Diuron	Phenylurea	100 μg	1774	1	0.06
EPTC	Thiocarbamate	10 μl	5630	4	0.07
Erbon	Phenoxyalkanoate	10 μl	2271	4	0.18
Fenac	Phenylacetic acid	50 μg	2283	2	0.09
Fenuron TCA	Phenylurea	1 μl	2010	1	0.05
Isocil	Substituted uracil	50 μg	2791	7	0.25
Linuron	Phenylurea	100 μg	2135	3	0.14
MH		25 μg	2411	3	0.12
Neburon	Phenylurea	100 μg	2404	3	0.12
Paraquat	Bipyridinium salt	20 μg	7981	8	0.10
Picloram	Picolinic acid	500 μg	1995	1	0.05
Propanil	Acylanilide	50 μg	3388	5	0.15
Propazine	Triazine	100 μg	2352	2	0.09
Solan	Acylanilide	25 μg	2830	0	0
TCA	Aliphatic acid	100 μg	2107	0	0
Triallate	Thiocarbamate	1 μl	2887	3	0.10
2,3,6-TBA	Benzoate	25 μg	2564	2	0.08
Trifluralin	Toluidine	25 μg	2570	2	0.08

Table V. The Effect of Selected Herbicides, Nonherbicidal Agricultural Chemicals, Nonagricultural Chemicals and Known Mutagenic Agents on the Induction of rII Mutants of T_4 Bacteriophage

Chemical	Amount of chemical added, μg	Mutation frequency, %[a] Experiment 1	Experiment 2
Control (no chemical)	0	0.00	0.37
5-Bromouracil	1000	...	3.33
2-Aminopurine	1000	...	0.37
Aminophylline	50	0.16	0.33
Caffeine	50	0.00	0.40
Colchicine	50	0.15	0.36
Amitrole	50	...	0.23
Maleic hydrazide	50	...	0.23
20–20–20 Garden Fertilizer	100	0.25	0.19
Aspirin	100	0.18	0.21
Pepper	100	0.12	0.31
Sodium chloride (iodized table salt)	100	0.97	0.29
Sodium chloride (analytical reagent grade)	100	...	0.33
Sucrose (table sugar)	100	0.26	0.29

[a] Mutation frequencies were calculated on the basis of total plaque number ranging from 2776 to 4521 for individual treatments.

Table VI. Effects of Known Mutagenic Chemicals and Herbicides on Reversion of Bacteriophage AP72 to T_4 Phenotype

Chemical	Amount of chemical added	Phage per[a] ml on B × 10^6	Phage per[a] ml on *E. coli* KB	T_4 per 10^6 AP72 phage	Relative mutagenic action
Thymine (control)	400 μg	225	210	0.93	1[b]
2-Aminopurine	1000 μg	103	410	3.98	4.28
5-Bromouracil	1000 μg	126	2711	21.52	23.14
Amiben	50 μg	82	15	0.18	0.19
Amitrole	400 μg	81	25	0.31	0.32
Atraton	500 μg	212	10	0.05	0.05
Atrazine	1000 μg	82	20	0.24	0.26
Bromacil	500 μg	172	15	0.09	0.10
CDAA	1.0 μl	158	0	0	0
CDEC	0.04 μl	124	35	0.28	0.30
2,4-D	50 μg	71	25	0.35	0.38
2,4-DEP	1.0 μl	108	70	0.65	0.70
Dicamba	2200 μg	135	30	0.22	0.24
Dalapon	500 μg	210	0	0	0
Dicryl	20 μg	145	25	0.17	0.18
DNOC	50 μg	84	50	0.60	0.65
Diphenamid	500 μl	79	20	0.25	0.27
Dipropalin	50 μg	84	10	0.12	0.13
Diquat	10 μg	116	95	0.82	0.88
EPTC	0.04 μl	175	50	0.29	0.31
Erbon	10 μl	121	150	1.24	1.33
Fenac	1300 μg	114	18	0.16	0.17
Fenuron TCA	0.04 μl	173	55	0.32	0.34
Isocil	1000 μg	84	40	0.48	0.52
Linuron	200 μg	179	50	0.28	0.30
MH	20 μg	116	50	0.43	0.46
Neburon	10 μg	216	65	0.30	0.32
Paraquat	10 μg	80	25	0.31	0.33
Picloram	6000 μg	155	75	0.48	0.52
Propanil	20 μg	124	35	0.28	0.30
Propazine	2000 μg	156	40	0.26	0.28
Solan	20 μg	134	45	0.34	0.37
TCA	100 μg	100	20	0.20	0.22
Triallate	0.04 μl	134	40	0.30	0.32
2,3,6-TBA	50 μg	78	40	0.51	0.55
Trifluralin	20 μg	142	90	0.63	0.68

[a] As measured by plaques on *E. coli* B and *E. coli* KB. [b] Relative rate of mutation due to the test compound over the spontaneous reversion rate, with thymine taken as 1.0.

induction, rII mutants appeared to the extent of about 2% of the population in the presence of 5-BU, while the spontaneous mutation rate was about 0.11%. The frequency of mutation to the rII type in the presence of several of the herbicides was slightly higher than the rate of spontaneous mutations, and therefore the results were evaluated statistically.

Two statistical tests were used to evaluate the data presented in Table IV. In the first statistical examination, properties of the binominal distribution were used to conduct tests comparing the mutation frequencies of various chemicals against the mutation frequency to a control to determine significant differences (Acheson, 1959). As a result of applying this test, 5-BU, isocil, bensulide, dicamba, and dipropalin showed a statistically significant increase in mutations over the control. Of these five, only 5-BU showed a marked significance. Isocil, bensulide, dicamba, and dipropalin were significantly different from the control at the 5% level and 5-BU was significantly different at the 1% level.

Those compounds, showing significance in the above binominal distribution test, were subjected to further analysis using the Poisson distribution test (Brownlee, 1960). The results of this statistical analysis clearly indicated that the mutagenic frequency was increased by 5-BU. The mutation frequencies associated with isocil, bensulide, dicamba, and dipropalin were of a magnitude that could be expected in about 7 to 9% of similar tests involving only untreated controls. Thus, the evidence for mutagenicity in any of the herbicides was slight, but it does indicate a need for additional research, with respect to these four.

The previously mentioned triazine derivatives were also evaluated for their ability to induce rII mutants of T_4 bacteriophage. The mutation frequencies were only slightly higher than the control and are similar to the results reported for the selected herbicides listed in Table IV, so that these derivatives also appeared nonmutagenic.

Evaluation of Common Herbicide Derivatives, and 3,3′,-4,4′-Tetrachloroazobenzene in the T_4 Bacteriophage/*E. coli* B Test System. Common aniline derivatives such as 3-chloroaniline, 4-chloroaniline, 2,4-dichloroaniline, and 3,4-dichloroaniline, as well as the suspected herbicide metabolite 3,3′,4,4′-tetrachloroazobenzene were also evaluated in the mutant induction system involving T_4 bacteriophage. These compounds did not show any evidence of mutagenicity. It should be noted that of the three concentrations of tetrachloroazobenzene evaluated, the 50-μg level showed marked inhibition, while the 25- and 12.4-μg levels, respectively, produced good plaque formation and nine mutant plaques.

Evaluation of Nonherbicidal Agricultural Chemicals and Nonagricultural Chemicals in the T_4 Bacteriophage/*E. coli* B Test System. Selected nonherbicidal agricultural chemicals and nonagricultural chemicals were evaluated for activity in inducing rII mutants. The purpose of these experiments was to provide data that would help in interpreting the significance of reversion rates observed with herbicides that were only slightly higher than those of the controls.

The procedure followed was the same as described previously and the results are listed in Table V. Of particular interest is the mutation frequency (0.97%) observed for sodium chloride in the first experiment. Ninety percent of the mutant plaques were on 2 of the 20 replications. We considered this to be the result of a mutational "hot spot," such as we have observed at times with untreated controls. During the first of these experiments no rII-type mutants were observed on the control plates. The mutation frequencies ob-

served with aminophylline, caffeine, and colchicine were also quite low. These three chemicals were examined because they have been directly associated with either mutagenesis (caffeine) or inhibition of cell division and mitosis (colchicine and aminophylline). The mutation frequencies observed with the remainder of the chemicals were all about the same as aminophylline, caffeine, and colchicine and considerably less than sodium chloride.

In the second experiment the known mutagens 5-BU and 2-AP, together with two additional herbicides, amitrole and maleic hydrazide, were included, along with all the chemicals of the first experiment.

It can be observed that the spontaneous mutation frequency in the second experiment was quite high at 0.37% (Table V). As expected, 5-BU yielded a high mutation frequency while 2-AP did not increase mutation frequency. Caffeine gave slightly higher frequencies than in the previous experiment but colchicine and aminophylline, which interfere with spindle fiber separation in the nucleus of plants, had little or no effect. All other chemicals in the second experiment were associated with mutation frequencies slightly lower than 0.37%. The mutation frequency observed for table salt in the second experiment, in contrast to that in the first experiment, did not exceed the spontaneous rate. Such materials as sucrose, fertilizer, aspirin, and pepper were associated with mutagenic frequencies of about the same size as that of herbicides in Table IV. We consider this to be evidence, although inconclusive, of a lack of mutagenic properties in the herbicides with respect to the microbial system we used.

Unexplained "hot spots," such as occurred with sodium chloride in the first of these experiments, render this test relatively insensitive for detection of very weak mutagenic action. Such "hot spots" have been observed in individual plates of untreated controls, and in individual plates treated with chemicals such as sodium chloride or herbicides.

Effects of Herbicides on the Reversion of Mutants of T_4 Bacteriophage. Table VI presents the effects of known mutagenic agents and selected herbicides on the reversion of the guanine–cytosine transition rII mutant, AP72. A strong mutagenic effect was observed with 5-BU, which is in agreement with the literature (Freese, 1959a,b). 2-AP was also mutagenic but was not nearly as active as 5-BU. When the results obtained with the herbicides were compared with those obtained with 5-BU and 2-AP, it appears that the herbicides were not mutagenic in the test system. Erbon produced a mutagenic action slightly higher than the control. However, when the mutant induction and reversion data were compared it was concluded that this chemical was not mutagenic. The plaques observed on *E. coli* KB were most likely the result of spontaneous mutations. Complementing the data presented in Table VI are the results obtained when the adenine–thymine transition rII mutant, N17, was used as the test organism (Table VII). For these experiments representative herbicides from each of the 12 major classes were evaluated. The herbicides continue to show a lack of mutagenesis, as observed with the rII mutant, AP72. The known mutagens 2-AP and 5-BU, gave a strong mutagenic response. However, the activity of 5-BU was less in this system than was observed in the AP72 system. N17 is a 5-BU induced rII mutant and according to the literature (Freese, 1959b) should respond less strongly to 5-BU than to 2-AP.

Table VII. Effects of Selected Herbicides on Reversion of Bacteriophage N17 to T_4 Phenotype

Test chemical	Amount of chemical added	Phage per[a] ml on *E. coli* B × 10^6	Phage per[a] ml of *E. coli* KB	T_4 per 10^6 N17 phage	Relative mutagenic action
Thymine (control)	50 μg	162	15	0.09	1.00[b]
2-Aminopurine	50 μg	167	110	0.66	7.17
5-Bromouracil	100 μg	188	45	0.4	2.60
Amiben	50 μg	121	10	0.08	0.87
Amitrole	400 μg	72	0	0	0
Atrazine	1000 μg	140	15	0.11	1.20
Bromacil	500 μg	100	10	0.10	1.09
CDAA	1.0 μl	99	10	0.10	1.09
2,4-D	50 μg	140	10	0.07	0.76
Dalapon	25 μg	110	5	0.05	0.54
DNOC	50 μg	69	5	0.07	0.76
Diquat	10 μg	178	5	0.03	0.33
EPTC	0.05 μl	85	5	0.06	0.65
Linuron	10 μg	90	5	0.06	0.65
Propanil	10 μg	87	0	0	0

[a] As measured by plaques on *E. coli* B and *E. coli* KB. [b] Relative rate of mutation due to the test compound over the spontaneous reversion rate, with thymine taken as 1.0.

DISCUSSION

Herbicides have been examined in a variety of microbial test systems to determine their mutagenicity. We were unable to detect any conclusive evidence of point mutations induced by any one of the 110 herbicides evaluated. These tests do not detect all forms of genetic damage and might fail to detect weakly mutagenic chemicals. However, our results do suggest that herbicides are not strongly mutagenic when compared to 5-BU, 2-AP, diethyl sulfate, and NG, which do induce various types of point mutation.

When herbicides were evaluated for the induction of mutations of T_4 bacteriophage (Table IV), several herbicides produced mutation frequencies that were in excess of the control. Lack of such effects in other test systems, smallness of the observed increases, higher spontaneous rates of mutation in other tests of the same system, and equal rates of mutations with nonherbicides in other tests of the same systems, however, indicate that the observed increases with the herbicides are within the normal range of the spontaneous rate.

MH has been implicated as a mutagenic chemical in microbial systems (Northrop, 1963). Our results indicate the contrary; mutation frequencies were only equal to or less than that of the controls. However, it should be mentioned that Northrop used a different system that involved the induction of virus production and there is some confusion as to whether this was a true mutation.

The objection can be made that results obtained with bacteria and bacteriophage cannot be extrapolated to cell tissue cultures or the whole organisms. Such an objection is questionable because one would expect that genetic changes of the point mutation type would be more likely to be revealed in microbial systems than in higher systems. Chromosome breaks, on the other hand, would not be detected in the systems we used. In initiating these experiments we were aware of the difficulties that would be encountered in extrapolating our results with microbial test systems to cell tissue culture systems or other higher order organisms. However, we believe that positive results can be extrapolated to a need for further research. We are confident in drawing conclusions from negative results, but nonetheless we believe that negative results can be useful in deciding where to place limited resources in future research.

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Evaluation of Bensulide for Mutagenic Properties in Microbial Test System

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The herbicide bensulide was evaluated for its ability to induce point mutations in the T_4 bacteriophage/*Escherichia coli* B system by measuring the frequency of inducing rII-type mutants of T_4 bacteriophage. Statistical analyses of the experimental data suggest that bensulide *per se* is not mutagenic in this particular microbial test system.

In a previous article, Andersen *et al.* (1972) reported on the evaluation of 110 herbicides for their ability to induce point mutations in one or more of four different microbial systems. With the exception of inconclusive evidence relating to four herbicides within one test of one of the test systems, the mutation rates of organisms treated with herbicides did not differ significantly from the spontaneous mutation rates.

In this one system, T_4 bacteriophage and *Escherichia coli* B, as the host bacterium, were used to detect chemically induced point mutations caused by selected herbicides. T_4 bacteriophage treated with each of these four herbicides exhibited mutation frequencies of 0.25 to 0.27%. In this particular test the spontaneous mutation rate was 0.11%. These data were subjected to statistical analysis and an increase from 0.11 to 0.25% was shown to be significant at the 5% level for one analysis method and at about the 10% level for a second method of analysis.

One of the herbicides that was associated with these slightly higher mutation rates was bensulide [*O,O*-diisopropyl phosphorodithioate *S*-ester with *N*-(2-mercaptoethyl)benzenesulfonamide]. Because of the serious implications of this finding, an extensive study was undertaken, employing the T_4 bacteriophage/*E. coli* B system to determine if bensulide is indeed mutagenic.

EXPERIMENTAL

Methodology. This test system involved the use of T_4 bacteriophage to detect chemically induced mutations of the rII type (Benzer, 1955) which might be caused by herbicides. The procedure followed was the same as used by Benzer and Freese (1958) in their study of the induction of mutations with 5-bromouracil. The T_4 bacteriophage and *Escherichia coli* B host are the same as described previously (Andersen *et al.*, 1972). For the purposes of this research program, 240 agar plates were used for each of the experiments to determine the following: spontaneous mutation rate (no test chemical); the mutation rate of a known mutagenic chemical (5-bromouracil, 5-BU); the mutation rate of bensulide; and the mutation rate of domestic table salt (a nonagricultural chemical control). Because of the limited solubility of bensulide in an aqueous medium, it was necessary to use 0.5 ml of acetone to aid in solubilization. In order to determine the effects of acetone on the test system, 240 tubes containing acetone as the test chemical were also evaluated.

Rather than running each of the 240 tube tests on separate days, a series of experiments were performed on different days involving 40 tubes containing no chemical, 40 tubes containing 5-BU, 40 tubes containing acetone, 40 tubes containing bensulide plus acetone, and 40 tubes containing table salt. The same T_4 bacteriophage stock suspension was used throughout all of these experiments.

Source of Herbicides and Other Chemicals. Bensulide was supplied by Stauffer Chemical Company and was the same supply that was used in the earlier study (Andersen *et al.*, 1972). At the initiation of this research program the purity of bensulide was checked and found to be 98–99% pure. 5-Bromouracil and acetone were obtained from P-L Biochemicals, Inc., and Baker and Adamson, Inc., respectively.

Statistical Analyses. The experimental series on rII mutant induction was conducted as a randomized complete block design. Blocks or replications were classified as days. The data were obtained as frequency counts per culture plate. These frequencies consisted of "normal" plaque and rII mu-

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