Metabolic Capabilities of the *Salmonella* Mutagenicity Test System

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^{14}C-labeled 2-acetylaminofluorene (AAF), carbaryl, DDT, and heptachlor were incubated with various components of the *Salmonella typhimurium* system used to detect mutagens and carcinogens. The bacteria, strain TA98, did not degrade any of the compounds except DDT during 48 h of incubation and then only when excess histidine was added to promote microbial growth. Approximately 20% of the DDT was converted to water-soluble materials and 1% to DDD. Addition of a rat liver fraction, 9000*g* solubles, to the system resulted in extensive metabolism of AAF (90%) and carbaryl (75%) after only 15 min. The metabolites were generally the same as those previously reported for intact animals, and, thus, the bacteria were exposed to products characteristic of mammalian metabolism. Heptachlor was rapidly converted to its epoxide form, 75% in 15 min, but the metabolic process essentially stopped at that point. The liver fraction did not alter DDT appreciably although its microbial degradation was the same as when the liver enzymes were omitted. With the latter two compounds, in vitro metabolism was not as extensive as that reported in in vivo studies, and, therefore, was not considered as being entirely representative of mammalian metabolism.

That a chemical may induce cancer in humans is the most feared of all toxic manifestations which may be attributed to an exogenous compound. This concern, magnified by the large number of chemicals to which man is exposed and the lengthy time required for adequate evaluation using classical animal studies, has prompted the development of rapid screening tests for estimating carcinogenic potential (Montesano et al., 1976). While such tests are not intended to replace animal feeding experiments, they may be extremely valuable in identifying those materials of highest priority for such experiments.

The *Salmonella typhimurium* mutagenic assay developed by Ames and co-workers (Ames et al., 1973a, b; Ames et al., 1975; McCann et al., 1975) is a significant contribution to the critical need for systems capable of expeditiously determining the potential of a chemical to induce cancer. This system utilizes histidine-dependent mutant strains of the bacteria that, under conditions of testing, flourish only if a chemical is added which induces a mutation allowing the bacteria to synthesize their own histidine. The assay allows the incorporation of mammalian microsomal enzymes so that chemicals requiring metabolic activation also may be detected. Excellent correlations have been demonstrated between the mutagenic activity and known carcinogens. However, some compounds, including the chlorinated insecticides used in the present study, are inactive in the Ames assay (Marshall et al., 1976) although they reportedly are carcinogenic in animals (Terracini et al., 1973; Thorpe and Walker, 1973; Tomatis and Turasov, 1975).

Intended as means of anticipating carcinogenic activity in animals, the bacterial screening system must meet certain basic requirements if the results are to be meaningful. The primary requirement is that the system differentiate between chemicals demonstrated to be carcinogenic and those shown to be noncarcinogenic in animal studies. Only then can results obtained with a compound of unknown carcinogenic activity be considered as highly indicative of what will occur in the living animal. For the most part, the Ames assay meets this basic criterion. Another requirement, and one which has not been established, is that the nature of the chemical to which the bacteria are exposed be the same as that to which animals are exposed. It is not unreasonable to expect that a compound may behave differently in the environment of the screening test than in the intact animal. The consequences may be either the formation of active metabolites, or derivatives, in the bacterial system which do not form in animals, or its failure to generate metabolites characteristic of that of mammalian metabolism.

The current study was designed to evaluate the chemical and biological stability of selected compounds in the Ames mutagenic/carcinogenic assay system under conditions as they prevail during standard testing procedures. Compounds selected were 2-acetylaminofluorene (AAF), carbaryl, DDT, and heptachlor, each of which has been similarly evaluated in animal systems. AAF is a known carcinogen and requires metabolic activation by liver microsomes to be active in the Ames assay (Ames et al., 1973a). Carbaryl is a carbamate insecticide which has not been implicated as a mammalian carcinogen, is inactive in the Ames assay, and whose metabolism in animals is well defined (Kuhr and Dorough, 1976). DDT and heptachlor, as previously stated, are chlorinated hydrocarbon insecticides indicted as mammalian carcinogens, but inactive in the Ames assay. These two materials were of particular interest since their inactivity in the bacterial system, even in the presence of rat liver microsomes, may be related to the manner in which they are metabolized.

MATERIALS AND METHODS

Chemicals. ^{14}C-labeled compounds were used in all experiments. Their common and chemical names and specific activities are as follows: AAF (2-acetylaminofluorene-9-^{14}C, 46.2 mCi/mmol); carbaryl (1-naphthyl-*1*-^{14}C *N*-methylcarbamate, 19.6 mCi/mmol); DDT (1,1,1-trichloro-2,2-bis(*p*-chlorophenyl-^{14}C-ethane, 2.7 mCi/mmol); and heptachlor (1,4,5,6,7,8,8-heptachlor-3a,4,7,7a-tetrahydro-4,7-methanoindene-^{14}C, 13.0 mCi/mmol). Each radioactive preparation was 98+% pure based upon TLC analysis and autoradiography. Authentic standards of certain known or suspected metabolites of the compounds (Table I) were used to assist in the identification of the products formed in the bacterial assay system.

Incubation. The radioactive compounds were incubated with the components of the *Salmonella* mutagenicity test prepared as described by Ames et al. (1975) for detecting carcinogens and mutagens. *S. typhimurium*, strain TA98, a frameshift mutant, was used as the test organism. This bacterial strain was kindly provided by Dr. B. N. Ames (Biochemistry Department, University of California, Berkeley). In brief, the standard procedure involves mixing the bacteria and test chemical in top agar con-

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Table I. Abbreviated Nomenclature and Chemical Names of Test Compounds and Metabolites Used in Study

Abbreviated nomenclature	Chemical name
Carbaryl	1-Naphthyl *N*-methylcarbamate
5,6-Dihydrodihydroxycarbaryl	5,6-Dihydrodihydroxy-1-naphthyl *N*-methylcarbamate
5,6-Dihydrodihydroxynaphthol	1-Hydroxy-5,6-dihydrodihydroxynaphthalene
N-Hydroxymethylcarbaryl	1-Naphthyl *N*-hydroxymethylcarbamate
4-Hydroxycarbaryl	4-Hydroxy-1-naphthyl *N*-methylcarbamate
5-Hydroxycarbaryl	5-Hydroxy-1-naphthyl *N*-methylcarbamate
1-Naphthol	1-Naphthol
AAF	2-Acetylaminofluorene
2-Aminofluorene	2-Aminofluorene
1-Hydroxy-AAF	2-Acetylamino-1-hydroxyfluorene
3-Hydroxy-AAF	2-Acetylamino-3-hydroxyfluorene
5-Hydroxy-AAF	2-Acetylamino-5-hydroxyfluorene
7-Hydroxy-AAF	2-Acetylamino-7-hydroxyfluorene
N-Hydroxy-AAF	*N*-Hydroxy-2-acetylaminofluorene
Heptachlor	1,4,5,6,7,8,8-Heptachlor-3a,4,7,7a-tetrahydro-4,7-methanoindene
Heptachlor epoxide	1,4,5,6,7,8,8-Heptachlor-2,3-epoxy-3a-4,5,7,7a-tetrahydro-4,7-methanoindan
DDT	1,1,1-Trichloro-2,2-bis(*p*-chlorophenyl)ethane
DDD	1,1-Dichloro-2,2-bis(*p*-chlorophenyl)ethane

taining just enough histidine to support several cell divisions, and pouring this over the bottom agar previously added to a 15 × 100 mm petri plate. The plates are then incubated for 48 h at 37 °C and the colonies counted to determine if the chemical was mutagenic. A rat liver preparation S-9 fraction and appropriate cofactors may be added to the top agar if metabolic activation of the chemical is to be considered.

For purposes of evaluating the stability of the radioactive chemicals in the mutagenic assay system, each compound (10^5 dpm) was added to 2 mL of top agar in 20 μL of acetone along with various components of the standard assay preparation. Glass petri plates were used to prevent absorption for DDT and heptachlor which occurred with plastic containers. Each experimental parameter investigated was conducted with sufficient plates to provide duplicate analysis at each designated time of incubation. Six separate sets of parameters were used for each compound. In one series of experiments, only the radioactive compound was added to the top agar. A second series of tests consisted of the compound and 10^9 bacteria per plate, while a third was identical except that 0.6 mg of histidine was also added. With this excess histidine, the bacteria grew profusely and their effect on the chemical could be determined. The fourth series of incubations consisted of plates containing top agar to which had been added the compound and a rat liver enzyme preparation. The latter was a mixture of cofactors and the 9000*g* solubles of rat liver homogenate (S9 RLH, 38 mg of liver equivalents/plate) prepared from animals injected 5 days prior to sacrifice with 500 mg/kg of Arochlor 1254 (Ames et al., 1975). A fifth series of experiments were conducted where the components of the top agar consisted of the compound, the liver homogenate, and the microbes; the sixth series was identical except for the addition of 0.6 mg of histidine. The periods of time the plates were incubated varied depending upon the rate of degradation of the compounds as determined in preliminary experiments, but did not exceed 48 h.

Analysis. The incubation medium from each plate was blended with 25 mL of distilled water and 25 mL of ethyl acetate. The homogenate was centrifuged for 30 min at 3300*g*, the supernatant transferred to a separatory funnel, and the organic phase collected. The agar and water were again extracted with 25 mL of ethyl acetate after which the organic extracts were combined and aliquots taken from each phase for radioassay by liquid scintillation counting. That radiocarbon in the ethyl acetate was referred to as organosolubles, that in the water as water solubles, and that remaining in the agar as unextractables.

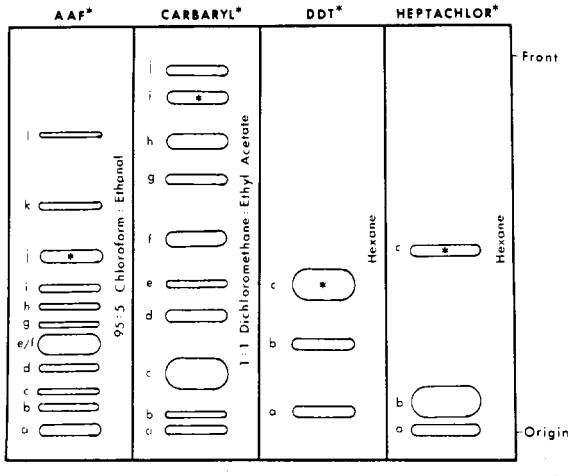

Figure 1. Drawings of thin-layer chromatograms showing those products formed from AAF, carbaryl, DDT, and heptachlor by the *Salmonella* mutagenic test system containing a rat liver homogenate. Additional TLC systems used for cochromatographic purposes are described in the text.

The organosoluble fraction was concentrated and subjected to TLC analysis.

Radioactive components of the organosoluble fractions were first resolved by one-dimensional silica gel TLC using those solvent systems shown in Figure 1. Autoradiography was used to locate the radioactive areas. Each of these areas was removed individually, the gel extracted with ethanol, and the material mixed with metabolite standards having similar R_f values. This mixture was then rechromatographed in various two-dimensional solvent systems to determine if the radioactivity was coincident with one of the standards. The basic two-dimensional system used to compare AAF standards with material from the first TLC was 95:5 chloroform–ethanol and 1:1 benzene–ethyl acetate. Additional systems used to verify cochromatography were: AAF, per se, 97:3 chloroform–methanol (R_f 0.27) and 1:1 benzene–ethyl acetate (R_f 0.35); 5- and 7-hydroxy-AAF, 7:3 ethyl acetate–chloroform (R_f 0.32, 0.38) and 1:1 benzene–ethyl acetate (R_f 0.21, 0.27); 3-hydroxy-AAF and *N*-hydroxy-AAF, 97:3 chloroform–methanol (R_f 0.10, 0.33) and 9:1 chloroform–ethanol (R_f 0.48, 0.56); 1-hydroxy-AAF, 1:1 benzene–ethyl acetate (R_f 0.41) and ethyl acetate (R_f 0.64); 2-aminofluorene, 97:3 chloroform–methanol (R_f 0.64) and 1:1 benzene–ethyl acetate (R_f 0.61). Carbaryl metabolites were identified on the basis of cochromatography of unknown and standard when developed two-dimensionally in 1:1 dichloro-

Table II. Fate of Radioactive AAF, Carbaryl, Heptachlor, and DDT in the *S. typhimurium* (strain TA98) Mutagenic Assay System without Rat Liver Homogenate

	% of radiocarbon added to medium/h of incubation											
	AAF			Carbaryl			Heptachlor			DDT		
Nature of radiocarbon	12	24	48	12	24	48	12	24	48	12	24	48
Organosolubles												
Parent compound	92.5	96.3	93.2	91.4	76.1	70.5	5.9	4.9	1.0	83.0	78.5	69.3
Other	0.8	0.8	0.6	6.8	13.1	19.4	11.6	16.9	8.5	0	0	0
Water solubles	0.2	0.2	0.1	1.3	2.1	5.3	2.0	1.6	1.8	0.2	0.5	0.3
Unextractables	0.1	0.1	0.1	0.5	1.9	4.8	0.8	0.8	1.2	1.5	3.9	5.4
Loss	6.4	2.6	6.0	0	6.8	0	79.7	75.8	87.5	15.3	17.1	25.0

[a] Data in this table were virtually identical with those obtained when the chemicals were incubated with agar alone, and, with the exception of DDT, the same as when excess histidine was added to promote microbial growth. After 48 h, the microorganism and histidine converted 21% of the [^{14}C]DDT to unextractables, 1.5% to water solubles, and 1% to DDD; the amount lost was 21.3%.

methane–ethyl acetate and 4:1 ether–hexane. This system was proven effective in resolving carbaryl metabolites in earlier studies (Dorough, 1971). DDT and heptachlor metabolites were resolved by developing the chromatograms in hexane as the first solvent system and 9:1 hexane–ether as the second system.

RESULTS AND DISCUSSION

Although the compounds under investigation were incubated with various components of the medium used in the Ames mutagenic assay, the results may be discussed relative to basically only two factors: the fate of the compounds without the addition of the S-9 liver enzyme and their fate when the liver fraction was added.

With the exception of DDT, the nature of the radiocarbon following incubation of each material with agar alone, with agar and bacteria, and with agar, bacteria, and excess histidine was the same (Table II). Even with DDT, the results were the same when the compound was incubated with just the agar and when the agar contained the bacteria. However, the addition of excess histidine to promote microbial growth did cause DDT to be degraded somewhat differently. The major difference was that the increased microbial activity converted 21% of the [^{14}C]-DDT to unextractable products after 48 h, whereas the agar alone and the mutant strain without excess histidine resulted in only 5.4% in this fraction. The actively growing bacteria also metabolized 1% of the DDT to a compound chromatographically identical with DDD which was not detected under the other conditions of incubation. These data demonstrate that the *S. typhimurium* strain TA98 mutants did not metabolize any of the compounds when their growth was limited, and only DDT when their growth was maximized by adding excess histidine to the system.

Without the liver enzymes, AAF was very stable in the assay medium during the 48-h incubation period (Table II). At each time of analysis, over 90% of the original radiocarbon remained as AAF, and total recovery was on the order of 95%. The small amount of radioactive material not identified as AAF consisted primarily of products remaining at, or very near, the TLC origin. Carbaryl was less stable in the medium than AAF, although total recovery of the radiocarbon was about the same. After 12 h of incubation, 91.4% of the carbaryl remained intact, but this declined to 70.5% after 48 h. There was a corresponding increase in other organosoluble products, almost all of which was 1-naphthol, as well as increased concentrations of the water-soluble and unextractable metabolites. Heptachlor was both unstable in the medium and apparently quite volatile. After only 15 min of incubation without the liver preparation, 40% of the added radiocarbon could not be accounted for in the extracts or agar. This loss increased to 87.5% after 48 h of incubation (Table II). That organosoluble material other than heptachlor remained at the TLC origin after development in hexane. DDT was converted to unextractable products by the agar medium to the extent of 5.4% of the original radiocarbon, and the loss after 48 h amounted to 25%. While the loss of heptachlor and DDT from the medium was of concern, no attempt was made to confirm the exact nature of the loss since, as shown below, it did not occur when the rat liver preparation was added to the agar.

AAF, carbaryl, and heptachlor were effectively degraded by the rat liver homogenate while DDT was relatively stable (Table III–V). With the exception of DDT, the bacteria did not influence the fate of the radioactive compounds added to the *Salmonella*/microsome system. The metabolic activity of the homogenate was readily evident by the conversion of 25.4% of the AAF to water-soluble products and 28.7% to unextractable material after 48 h of incubation (Table III). Approximately 25% of the AAF was converted to 11 organosoluble metabolites of a nature generally comparable to those formed in animals. In vivo mammalian metabolism of AAF includes deacetylation, hydroxylation of the 1, 3, 5, 6, 7, and 8 positions of the ring, and N-hydroxylation (Miller et al., 1960; Weisburger and Weisburger, 1956; Weisburger et al., 1956, 1959). Relative concentrations of metabolites detected in the Ames in vitro system were similar to the free urinary products from rats fed AAF (Miller et al., 1960; Weisburger and Weisburger, 1956). That is, low concentrations of 1- and 3-hydroxy-AAF, 2-aminofluorene, and AAF were detected with significantly higher concentrations of 5- and 7-hydroxy-AAF. Organosoluble metabolites produced in the Ames test by the liver homogenate included 1-, 3-, 5-, and 7-hydroxy-AAF and 2-aminofluorene (Table III). Concentrations of less than 1% each were obtained with 1- and 3-hydroxy-AAF and 2-aminofluorene after 48 h of incubation, while levels of 5- and 7-hydroxy-AAF were 3.1 and 7.2% of the added radioactivity, respectively.

An important metabolite not detected in the free form in the Ames assay system or in rat urine was *N*-hydroxy-AAF. This metabolite, believed to be a precursor to the ultimate carcinogen, accounted for up to 15% of the dose after 18 weeks of continued feeding of AAF to rats (Miller et al., 1960). It existed entirely as the glucuronide conjugate. Free *N*-hydroxy-AAF would not necessarily be expected in the Ames system since it would probably be converted to the glucuronide conjugate or to AAF-*N*-sulfate, a proposed active metabolite (Miller, 1970). Although conjugated metabolites of AAF were not demonstrated experimentally in this study, their presence was indicated by the production of large quantities of water soluble and unextractable metabolites. While several

Table III. Fate of [^{14}C]AAF in the *S. typhimurium* (strain TA98) Mutagenic Assay System Containing the 9000*g* Solubles of a Rat Liver Homogenate

Nature of radiocarbon	% of [^{14}C]AAF added to medium/h of incubation					
	Control[a]	.25	12	24	48	48 + H[b]
Unextractables	0.2	8.4	24.5	25.4	28.7	31.4
Water solubles	0.3	5.5	26.0	25.8	25.4	23.9
Organosolubles	99.1	75.9	36.6	35.0	34.5	35.6
(a) Unknown I-A[c]	0	4.4	7.8	7.2	3.7	3.8
(b) Unknown II-A	0	12.3	4.6	3.1	2.3	2.7
(c) Unknown III-A	0	3.6	2.8	2.4	2.2	2.9
(d) Unknown IV-A	0	1.6	3.7	3.5	3.9	3.5
(e) 7-Hydroxy-AAF	0	26.1	4.3	5.5	7.2	8.9
(f) 5-Hydroxy-AAF	0	11.2	1.8	2.3	3.1	3.2
(g) Unknown V-A	0	1.0	1.1	0.8	1.0	0.6
(h) 3-Hydroxy-AAF	0	3.2	0.6	0.7	0.4	0.3
(i) 1-Hydroxy-AAF	0.1	0.8	0.4	0.4	0.4	0.5
(j) AAF	98.7	9.9	9.2	8.5	9.8	8.7
(k) Unknown VI-A	0.1	0.7	0.2	0.3	0.3	0.4
(l) 2-Aminofluorene	0.2	1.1	0.1	0.3	0.2	0.1
Total recovery	99.6	89.8	87.1	86.2	88.6	90.9

[a] Same as the 0.25-h sample except that liver homogenate was boiled. [b] Same as the 48-h sample except that 0.6 mg of histidine was added so that active microbial growth occurred. [c] Radiocarbon remaining at TLC origin.

Table IV. Fate of the Carbamate Insecticide [^{14}C]Carbaryl in the *S. typhimurium* (strain TA98) Mutagenic Assay System Containing the 9000*g* Solubles of a Rat Liver Homogenate

Nature of radiocarbon	% of [^{14}C]carbaryl added to medium/h of incubation								
	Control[a]	0.25	1	3	6	12	24	48	48 + H[b]
Unextractables	0	12.2	14.9	17.5	17.3	20.0	20.4	17.4	15.1
Water solubles	0	20.4	24.4	25.8	27.4	26.6	28.6	27.5	29.0
Organosolubles	95.5	62.2	50.0	46.0	41.5	40.1	41.2	41.9	46.1
(a) Unknown I-C[c]	0.2	2.1	3.1	1.9	2.0	3.0	2.4	3.2	3.8
(b) Unknown II-C	0	0.9	0.8	0.9	0.9	0.9	0.8	1.0	1.5
(c) 5,6-Dihydrodihydroxycarbaryl	0	14.3	16.4	15.2	17.6	15.3	16.1	16.4	16.3
(d) 5,6-Dihydrodihydroxynaphthol	0	1.1	1.3	1.7	2.3	3.4	5.7	6.3	5.4
(e) Unknown III-C	0	1.2	1.4	1.7	2.3	1.5	1.5	1.1	2.4
(f) *N*-Hydroxymethylcarbaryl	0	5.9	4.8	2.5	1.5	1.3	0.7	0.7	0.7
(g) 4-Hydroxycarbaryl	0	5.9	2.7	2.8	3.7	2.3	1.9	2.4	3.4
(h) 5-Hydroxycarbaryl	0	3.8	3.2	3.1	3.3	2.6	1.3	1.6	1.8
(i) Carbaryl	94.7	25.6	13.2	15.2	6.8	8.4	9.4	7.8	10.2
(j) 1-Naphthol	0.6	1.4	3.1	1.0	1.1	1.4	1.4	1.4	0.6
Total recovery	95.5	94.8	89.3	89.3	86.2	86.7	90.2	86.8	90.2

[a] Same as the 0.25-h sample except that liver homogenate was boiled. [b] Same as the 48-h sample except that 0.6 mg of histidine was added so that active microbial growth occurred. [c] Radiocarbon remaining at TLC origin.

Table V. Fate of the Chlorinated Insecticides [^{14}C]DDT and [^{14}C]Heptachlor in the *S. typhimurium* (strain TA98) Mutagenic Assay System Containing the 9000*g* Solubles of a Rat Liver Homogenate

Nature of radiocarbon	% of ^{14}C insecticide added to medium/h of incubation					
	Control[a]	0.25	12	24	48	48 + H[b]
		DDT				
Unextractables	1.4	2.1	4.7	8.3	11.8	20.6
Water solubles	0.2	0.7	1.4	1.9	1.7	0.8
Organosolubles	91.4	86.6	93.2	88.2	79.2	72.8
(a) Unknown I-D	0	0	0	0.5	0.7	0.9
(b) DDD	0	0	0	0	0	3.5
(c) DDT	91.4	86.6	93.2	87.7	78.5	68.4
Total recovery	93.0	89.4	99.3	98.4	92.7	94.2
		Heptachlor				
Unextractables	0.1	1.6	5.8	7.2	9.7	9.4
Water solubles	0.3	3.1	8.9	9.9	10.2	9.9
Organosolubles	94.9	89.5	81.4	80.2	72.6	73.1
(a) Unknown I-H[c]	1.0	2.3	8.2	5.7	13.9	10.6
(b) Heptachlor epoxide	0.7	74.7	69.2	70.2	58.0	61.7
(c) Heptachlor	93.2	12.5	4.0	4.3	0.7	0.8
Total recovery	95.3	94.2	96.1	97.3	92.5	92.4

[a] Same as the 0.25-h sample except that liver homogenate was boiled. [b] Same as the 48-h sample except that 0.6 mg of histidine was added so that active microbial growth occurred. [c] Radiocarbon remaining at TLC origin.

products produced by liver homogenate in the Ames assay system remain unidentified, the characterization of the major metabolites and their close quantitative correlation to the in vivo metabolism of AAF suggest that the mutagenic test with microsomes closely simulated mammalian metabolism.

The metabolism of carbaryl by the liver enzymes was extensive (Table IV). As with AAF, carbaryl was rapidly

metabolized into water-soluble (27.5%) and unextractable products (17.4%). Nine organosoluble metabolites were resolved by TLC. Unknown I-C was radioactive material which remained at the TLC origin. This material probably consisted of conjugated products partially extracted from the water phase. Although an authentic reference standard was unavailable for verification, unknown II-C demonstrated the same TLC characteristics as 3,4-dihydrodihydroxy-1-naphthyl *N*-methylcarbamate. This compound was reported as a metabolite in milk from a lactating cow fed [^{14}C]carbaryl (Dorough, 1971). Unknown III-C did not correspond to any of the available reference standards and was not present in high enough concentration to allow identification.

The major free metabolite produced by the liver homogenate was 5,6-dihydrodihydroxycarbaryl. After 48 h of incubation, this product represented 16.4% of the applied radiocarbon while its hydrolyzed derivative, 5,6-dihydrodihydroxynaphthol, accounted for 6.3%. The 4-hydroxy, 5-hydroxy, and *N*-hydroxymethyl derivatives of carbaryl were the only metabolites which declined in concentration after the initial incubation period of 15 min. Metabolism of carbaryl within the Ames mutagenic test system appears representative of the mammalian metabolism of this carbamate. The compound was rapidly metabolized primarily by oxidative and hydrolytic processes to products which were probably metabolized further to glucuronide and sulfate conjugates as reported for various animal systems (Dorough, 1967, 1971).

Both chlorinated hydrocarbons tested in this study are nonelectrophilic compounds, and one would assume that DDT and heptachlor are not direct-acting carcinogens. The evidence of species specificity to tumor induction also indicates the requirement for activation (Agthe et al., 1970; Gingell and Wallcave, 1973). Metabolic activation in the Ames system may be imperative before DDT and heptachlor are detected as mutagens.

A major pathway of degradation of DDT in man involves dehydrochlorination to form DDE. This metabolite is very stable and is slowly eliminated from the body. Mammalian metabolism also involves reductive dechlorination to DDD which is further metabolized to DDA. DDD and conjugates of DDA are major components of the animal excreta (Brooks, 1974). Incubation of DDT with the liver homogenate and other components of the Ames system revealed that DDT was not converted to DDE and, in fact, was very resistant to attack of any type by the microsomal enzymes (Table V). As stated earlier, microbial degradation was responsible for the conversion of a small percentage of DDT to DDD and other unidentified metabolites, but 78.5% of the radiocarbon still remained in the form of DDT after 48 h of incubation. These data support the supposition of McCann and Ames (1976) that DDT is inactive in the Ames assay because of the inability of the microsomes to metabolically activate the insecticide. However, an active metabolite must be isolated from intact animals before this can be verified.

Heptachlor behaved differently from DDT when incubated with the liver homogenate. Within 0.25 h of incubation, 74.7% of heptachlor was converted to the epoxide derivative (Table V). This reaction occurs in most living organisms and in the environment (Brooks, 1974). However, heptachlor epoxide is inactive in the *Salmonella* mutagenic test system (Marshall et al., 1976). Prolonged incubation up to 48 h resulted in a gradual decrease in the concentration of the epoxide with a proportional increase in polar products. The increase in polar products may result from hydroxylation, followed by conjugation, of heptachlor or the epoxide at the allylic chlorine atom. Such hydroxylation was demonstrated in vivo to form a hydroxy epoxide which was susceptible to desaturation within the ring. Reports of rabbit in vivo metabolism have also indicated the hydrolysis of the epoxide to form a diol derivative (Brooks, 1974). The hydroxylated products were not detected in the present study but may have existed as conjugates in the water phase of the medium extracts. Regardless of the nature of the metabolites, they are either inactive or produced in insufficient quantities to induce a mutation in the *Salmonella* test strains (Marshall et al., 1976).

To this point, only brief mention has been made relative to the influence of incubation time on the microsomal metabolism of the compounds used in the present investigation. The reason is simply that very little metabolism occurred with any of the compounds after the initial 15 min of incubation. Analyses of carbaryl incubations of 1, 3 and 6 h showed that the degree of metabolism was only slightly greater at these times than that observed after 15 min and that this increase occurred primarily during the first hour of incubation (Table IV). A similar pattern was exhibited by AAF when subjected to identical tests. While certain of the free AAF metabolites decreased significantly in concentration between 15 min and 1 h, a rather stable situation existed thereafter. DDT and heptachlor epoxide concentrations continued to decline after 15 min but at such a slow rate that enzyme involvement was questionable. These data suggested that the microsomal enzymes were active in the test medium for about 1 h as they are in typical buffered preparations. This is not consistent with earlier reports that the microsomes retained their activity (activation of vinyl chloride) for 9 h at 37 °C when added to the agar overlay (Bartsch et al., 1975) and that the agar acts to stabilize the immobilized enzymes (Ames, 1975). Using [^{14}C]carbaryl as a test compound, we established that the enzymes were just as active after incubation for 9 h at 37 °C in the agar medium as they were at zero time. Based on these results and those of the vinyl chloride study, it appears that while the microsomes remain viable for prolonged periods, metabolites are produced almost immediately or not at all. Those which are produced may be inactive whereas their subsequent metabolism could lead to mutagenic compounds. Therefore, if a compound is not metabolically activated by the liver fraction in the standard *Salmonella* mutagenic test, one can not conclude that similar results would occur in the intact animal where more complete metabolic processes may result in additional metabolites and/or where residues of active metabolites may accumulate to much higher levels.

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Mutagenic Activity of Thiocarbamate Herbicides in *Salmonella typhimurium*

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Three thiocarbamate herbicides, diallate (*S*-(2,3-dichloroallyl)diisopropylthiocarbamate), triallate (*S*-(2,3,3-trichloroallyl)diisopropylthiocarbamate), and CDEC (2-chloroallyl-*N*,*N*-diethyldithiocarbamate), were evaluated for their ability to induce mutations in four histidine-requiring strains of *Salmonella typhimurium* (TA 100, TA 1535, TA 98, and TA 1538) with and without a rat liver microsomal activation system (Ames test). These herbicides were mutagenic in TA 100 and TA 1535 (base-pair substitution mutants) only in the presence of the liver microsomal preparation, indicating that the chemicals require metabolic activation for their conversion into active mutagens. None of the herbicides caused mutations in strains TA 98 and TA 1538 (frameshift mutants). Diallate was considerably more potent than triallate or CDEC, showing mutagenic activity at 1 μg/plate.

Diallate (*S*-(2,3-dichloroallyl)diisopropylthiocarbamate), triallate (*S*-(2,3,3-trichloroallyl)diisopropylthiocarbamate), and CDEC (2-chloroallyl-*N*,*N*-diethyldithiocarbamate) are used as preemergence herbicides for controlling weeds in certain vegetable and field crops (Weed Science Society of America, 1974). The annual consumption of diallate, triallate, and CDEC in 1975 was reported to be 0.3, 1.3, and 0.1 million pounds, respectively (Stanford Research Institute, 1976). Man may be exposed to these chemicals through occupational exposure or by consuming food containing residues of the chemicals. Although the toxicity and biochemical effects of these herbicides in plants have been reported (Ashton and Crafts, 1973; Fang, 1975), an extremely limited amount of information is available on their carcinogenicity or mutagenicity. Innes et al. (1969) reported that diallate was carcinogenic in mice. In view of the fact that most carcinogens are also mutagens (McCann et al., 1975a; McCann and Ames, 1976), it was of interest to determine whether or not the carcinogenic diallate and the structurally related triallate and CDEC were capable of inducing mutations.

A simple, rapid, sensitive, and economical method for detecting potential mutagens is the microbial mutagenicity assay developed by Ames et al. (1973). Anderson et al. (1972) evaluated 110 herbicides including diallate, triallate, and CDEC for their ability to induce point mutations in eight histidine-requiring strains of *Salmonella typhimurium*. They reported that none of the herbicides induced mutations in the test system. In their studies the bacterial cells were treated with the herbicides in the absence of a liver microsomal activation system. It is now well established that many chemicals are not mutagenic per se but require metabolic activation of the expression of their mutagenic activity (Ames et al., 1973; Ong and Malling, 1975; Malling, 1971). Such chemicals will not be detected by mutagenic screening techniques which use microorganisms to detect genetic damage unless mammalian metabolism is first allowed to act on the chemicals. It is quite possible that the lack of mutagenicity observed in *Salmonella* in the studies by Anderson et al. (1972) may have been due to the fact that the herbicides were not metabolized to the active form by bacterial enzymes. In order to test this possibility, we have investigated the mutagenicity of diallate, triallate, and CDEC by incubating them with *Salmonella* strains in the presence of rat liver microsomes. We show here that these herbicides which are nonmutagenic in *Salmonella* without metabolic activation can be converted to mutagens by a liver microsomal enzyme system.

MATERIALS AND METHODS

Bacterial Strains. The *Salmonella typhimurium* strains (TA 1535, TA 1538, TA 98, TA 100) used for the detection of mutagens were obtained from Dr. Bruce Ames, University of California at Berkeley. All the strains are histidine-dependent and are induced to mutate back to histidine-independence by particular mutagens. These

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