Adenosine Triphosphatase Activity in Liver, Intestinal Mucosa, Cloacal Bladder, and Kidney Tissue of Five Turtle Species Following *in Vitro* Treatment with 1,1,1-Trichloro-2,2-bis(*p*-chlorophenyl)ethane (DDT)

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Liver, intestinal mucosa, cloacal bladder, and kidney tissue were removed from five turtle species and assayed for adenosine triphosphatase (ATPase) activity following treatment with various concentrations of DDT for 30 min. These data indicate that the total (Na^+,K^+,Mg^{2+})-, Mg^{2+}-, and (Na^+,K^+)-dependent ATPases in the tissues of the five turtle species studied were significantly inhibited by 53 μM DDT. Treatment with 5.3 μM DDT caused some inhibition and stimulation. These results suggest that active transport may be severely affected by the action of DDT upon these ATPase systems.

Interest in the environmental impact of organochlorine insecticides has led to numerous studies concerning the effect of these compounds on active transport across cellular membranes.

One of the initial *in vitro* demonstrations of an enzymatic active transport system that involved adenosine triphosphate (ATP) was reported by Skou (1957), who isolated (Na^+,K^+)-dependent adenosine triphosphatase (ATPase) from crab nerve membranes. This ATPase was stimulated by Na^+ and K^+ and was inhibited by ouabain, a cardiac glycoside. Caldwell *et al.* (1960) found that potassium cyanide could effectively inhibit the active transport system in squid axons, but injection of ATP through the membrane into the intracellular portion of the nerve restored active transport.

One of the initial efforts to specifically explain the action of DDT in terms of active transport was by Matsumura *et al.* (1969). By using differential centrifugation techniques, they isolated various nerve components of rat brain and localized the source of ATPase sensitive to DDT. Their results indicated that (Na^+,K^+,Mg^{2+})-dependent ATPase in the rat brain is specifically sensitive to DDT. They suggested that DDT is causally related to disruption of ion transport mechanisms in the nervous system *in vivo*. The molecular aspects of the reaction through which DDT caused such disruption of the membrane function were unknown. Matsumura and Narahashi (1971) attempted to correlate the degree of ATPase inhibition with electrophysiological symptoms of DDT poisoning in the lobster nerve. They concluded that DDT-induced membrane conductance changes might possibly be related to ATPase inhibition by DDT *in vitro*. It was found that DDT-sensitive ATPase was not identical with the ouabain-sensitive (Na^+,K^+)-dependent ATPase. Chemicals known to induce nerve conductance changes similar to DDT were found to be potent ATPase inhibitors.

Interest in mechanisms of reciprocal transfer of Na^+ and K^+ across the plasma membrane of individual cells caused Jampol and Epstein (1970) to investigate the specific activity of (Na^+,K^+)-dependent ATPase in gill, intestine, and kidney tissues of the American eel, *Anguilla rostrata*. Their data indicate that (Na^+,K^+)-dependent ATPase plays an important role in active transport of sodium across epithelial membranes. Using similar methods, Janicki and Kinter (1971) conclusively showed that DDT inhibits (Na^+,K^+,Mg^{2+})-dependent ATPase engaged in active sodium transport functioning to maintain tissue osmolarity. In their study, 1–10 ppm of DDT in the *in vitro* assay caused up to 40% inhibition of the enzyme. They also found that cyclohexanone (a commercial DDT solvent) completely inhibited (Na^+,K^+,Mg^{2+})-dependent ATPases.

In long-term exposure of organochlorine compounds *in vivo*, (Na^+,K^+,Mg^{2+})-dependent ATPases were inhibited in several fish species (Koch *et al.*, 1972). In some cases, notably at the lower concentrations of DDT, erratic stimulation occurred. Stimulation was most pronounced in kidney and liver tissues.

The purpose of this study was to determine the effect of various concentrations of DDT upon the ATPase systems in five turtle species.

METHODS AND MATERIALS

The activity of adenosine triphosphatase (ATPase) was determined in turtle tissues by measuring the amount of inorganic phosphate produced when adenosine triphosphate was converted to adenosine diphosphate.

Five turtle species were assayed for ATPase activity: the common snapping turtle, *Chelydra serpentina;* map turtle, *Graptemys geographica;* red-eared turtle, *Pseudemys scripta;* softshell turtle, *Trionyx spinifer;* and midland painted turtle, *Chrysemys picta*. All species were trapped in middle and west Tennessee. Each turtle was placed in plastic swimming pools 1.5 m in diameter. Water levels were maintained at 5 cm in the storage pools. Room temperature ranged from 23 to 27°. Animals were exposed to a diurnal cycle of natural lighting in conjunction with a timed artificial lighting system. All map turtles assayed were females; all of the remaining specimens assayed were males.

Each turtle was anesthetized with ethyl ether until it was limp (30–60 min). The plastron was removed with a Weber bone saw. Portions of the liver, kidney, cloacal bladder, and intestinal mucosa were removed and placed on cold aluminum foil. Each tissue sample was immediately weighed and added to a cold solution containing 0.25 *M* sucrose, 0.005 *M* disodium ethylenediaminetetraacetic acid, and 0.003 *M* histidine buffer (pH 7.4) to yield a 5% w/v concentration (Janicki and Kinter, 1971). Tissues were homogenized in a Ten Broeck glass homogenizer in ice. Glassware was washed immediately after each determination with distilled deionized water and the Fiske and SubbaRow (1925) reagents. After each fifth determination, the glassware was washed with hot 10 *N* HCl and rinsed. A 0.2-ml aliquot of the tissue homogenate was added to 4.25 ml of the incubation media containing 20 m*M* histidine buffer (adjusted to pH 7.4), 100 m*M* NaCl, and 20 m*M* KCl (Janicki and Kinter, 1971). Each sample also contained either 0.25 ml of 100% *N,N*-dimethylformamide (DMF) or DDT concentrations of 10^{-3}, 10^{-4}, and 10^{-5} *M* in DMF. DMF at this concentration was found to have no effect on ATPase activity. Samples were assayed

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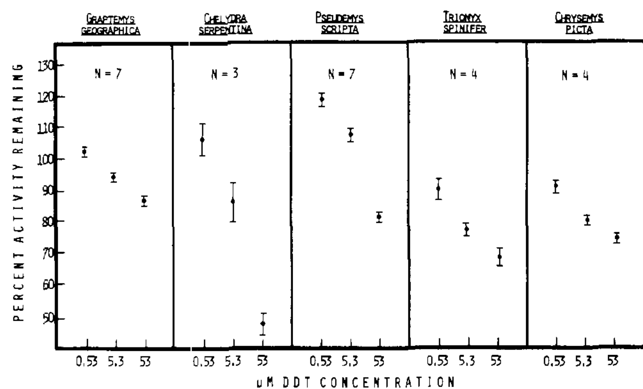

Figure 1. Effect of *in vitro* DDT treatment (30 min) on the total (Na^+,K^+,Mg^{2+})-dependent ATPase in the intestinal mucosa of five turtle species. Bars represent ± one standard error; N = number of turtles assayed.

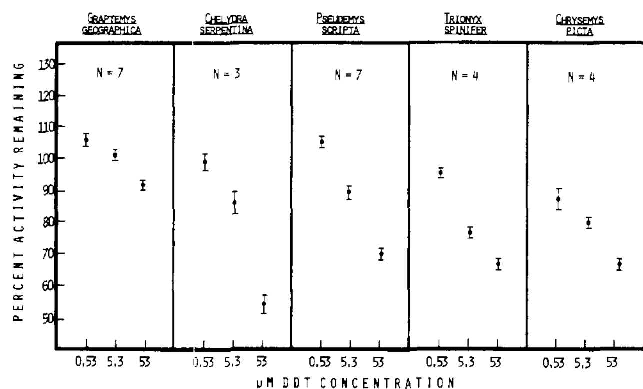

Figure 2. Effect of *in vitro* DDT treatment (30 min) on the total (Na^+,K^+,Mg^{2+})-dependent ATPase in the cloacal bladder of five turtle species. Bars represent ± one standard error; N = number of turtles assayed.

in triplicate. Samples were incubated for 30 min to allow the DDT to pervade the homogenate. The reaction was initiated by addition of 50 µl of 100 mM Na_2ATP and 100 mM $MgCl_2$ and continued with agitation for 30 min at 24°. This gave DDT concentrations of 53, 5.3, and 0.53 µM in the reaction mixtures. ATPase activity was terminated by the addition of 1 ml of ice-cold 30% trichloroacetic acid. Samples were then transferred for 30 min to a refrigerator (5°) to allow complete precipitation of the homogenate proteins. The precipitate was sedimented in an International Clinical Centrifuge at 3000 rpm for 3 min.

DDT was extracted from each sample using equal amounts of ice-cold hexane to preclude density interference in the colorimetric determination of inorganic phosphate by insoluble suspended crystals. Samples were stored overnight at 0° to facilitate separation of the homogenate from the hexane. The unfrozen top layer (hexane-DDT) was decanted and the homogenate thawed to room temperature.

Inorganic phosphate produced as a result of the cleavage of ATP was measured by the method of Fiske and SubbaRow (1925) as modified by Bartlett (1958). Color development proceeded at room temperature for 10 min. The optical density of each sample was measured with a Beckman Spectronic 70 spectrophotometer at 660 nm.

The ATPase activity was determined for total (Na^+,K^+,Mg^{2+})-dependent ATPase and Mg^{2+}-dependent ATPase (ouabain used to inhibit (Na^+,K^+)-dependent ATPase). Total ATPase activity minus Mg^{2+}-dependent ATPase activity gave the (Na^+,K^+)-dependent ATPase activity. A comparison of ATPase activity between species was based on homogenate protein (method of Lowry *et al.*, 1951). Statistical analysis was by the t test.

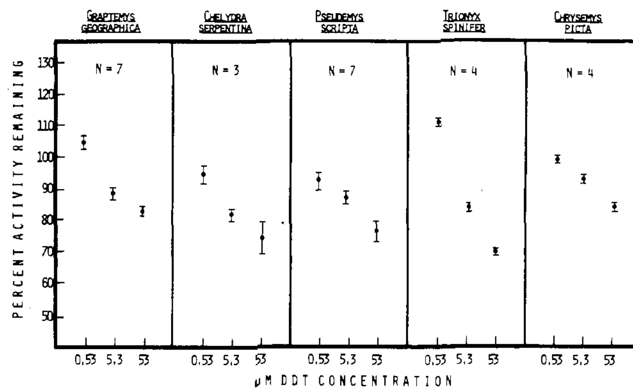

Figure 3. Effect of *in vitro* DDT treatment (30 min) on the total (Na^+,K^+,Mg^{2+})-dependent ATPase in the kidney of five turtle species. Bars represent ± one standard error; N = number of turtles assayed.

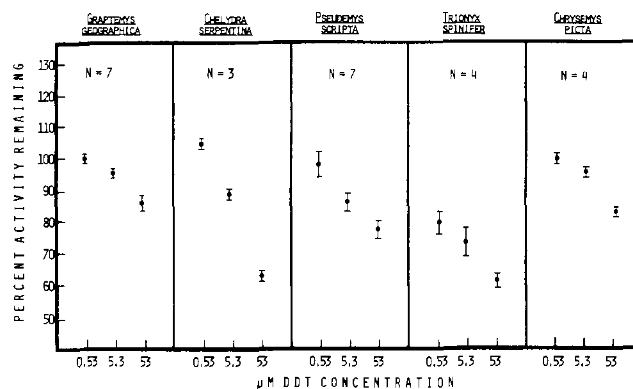

Figure 4. Effect of *in vitro* DDT treatment (30 min) on the total (Na^+,K^+,Mg^{2+})-dependent ATPase in the liver of five turtle species. Bars represent ± one standard error; N = number of turtles assayed.

RESULTS AND DISCUSSION

Utilizing procedures adapted from current literature, numerous preliminary ATPase determinations resulted in what appeared to be no enzyme activity. Concentrations of ATP greater than that required for the ATPase assay were found to inhibit the development of the color complex. Inhibition was probably due to the molybdate-catalyzed hydrolysis of excess ATP during color development (Marsh, 1958).

In all species studied and in all tissues assayed, inhibition of the total (Na^+,K^+,Mg^{2+})-dependent ATPase occurred at 53 µM DDT (Figures 1–4). The greatest inhibition of ATPase among all organs of the five species was observed in the intestinal mucosa homogenates of *Chelydra serpentina*, which retained 48% of its original total ATPase activity (Figure 1). The greatest stimulation of total ATPase activity in the five species occurred in the intestinal mucosa of *Pseudomys scripta* at 0.53 µM DDT. The greatest inhibition by DDT of total ATPase from the cloacal bladder was found in *Chelydra serpentina* (Figure 2). The total ATPase activity in the cloacal bladder of *Chelydra serpentina, Pseudemys scripta, Trionyx spinifer,* and *Chrysemys picta* was also significantly inhibited at 5.3 µM DDT. Only *Chrysemys picta* showed significant inhibition of total ATPase activity at 0.53 µM DDT in the cloacal bladder. The greatest inhibition of total ATPase activity in the kidney was in *Trionyx spinifer* at 53 µM DDT (Figure 3). Inhibition of total ATPase activity in liver tissue was greatest in *Trionyx spinifer* at all concentrations of DDT (Figure 4).

The highest Mg^{2+}-dependent ATPase levels found in this study were in the cloacal bladder tissue of *Graptemys*

Table I. Effect of *in Vitro* DDT Treatment (30 min) on the Mg^{2+}-Dependent ATPase Activity in Liver (L), Intestinal Mucosa (I), Cloacal Bladder (B), and Kidney (K) Whole Homogenates from Five Turtle Species

			μg of phosphate/mg of protein per 30 min[a]			
				DDT concentration		
Species	*N*	Organ	Control	53 μM	5.3 μM	0.53 μM
Graptemys geographica	7	L	49.71 ± 0.44	41.89 ± 0.69**	57.55 ± 0.76**	58.89 ± 0.95**
		I	50.41 ± 0.64	43.86 ± 0.33**	45.86 ± 0.42**	53.76 ± 0.36**
		B	63.12 ± 0.44	59.48 ± 0.91**	64.10 ± 0.28	75.11 ± 0.27**
		K	36.67 ± 0.51	29.21 ± 0.32**	47.37 ± 0.75**	47.76 ± 0.76**
Chelydra serpentina	3	L	9.83 ± 0.04	6.64 ± 0.17**	12.86 ± 0.27**	14.68 ± 0.50**
		I	37.33 ± 0.73	33.47 ± 0.05**	33.68 ± 0.80**	35.68 ± 0.63
		B	16.32 ± 0.62	9.97 ± 0.30**	13.88 ± 0.41**	21.49 ± 0.30**
		K	10.94 ± 0.94	6.62 ± 0.11**	12.11 ± 0.14*	19.19 ± 0.32**
Pseudemys scripta	7	L	27.63 ± 0.31	25.51 ± 0.24**	28.21 ± 0.44	28.33 ± 0.18*
		I	25.68 ± 0.56	22.97 ± 0.38**	31.76 ± 0.40**	33.53 ± 0.70**
		B	50.09 ± 1.00	42.76 ± 0.66**	66.46 ± 0.79**	54.29 ± 0.90*
		K	28.11 ± 0.25	21.25 ± 0.42**	23.35 ± 0.19**	27.19 ± 0.29*
Trionyx spinifer	4	L	31.73 ± 0.92	30.35 ± 1.10	30.78 ± 1.31	33.56 ± 0.72*
		I	32.22 ± 0.38	25.07 ± 0.32**	29.15 ± 0.20**	34.32 ± 0.56**
		B	17.89 ± 0.16	12.71 ± 0.23	17.96 ± 0.33	29.86 ± 0.36**
		K	37.51 ± 0.21	31.78 ± 0.26**	30.95 ± 0.47**	42.22 ± 1.84**
Chrysemys picta	4	L	49.14 ± 0.77	54.76 ± 0.39**	55.86 ± 0.40**	59.35 ± 0.27**
		I	32.70 ± 1.20	33.13 ± 0.42	43.07 ± 0.49**	36.89 ± 1.30**
		B	46.83 ± 0.36	40.20 ± 0.30**	46.46 ± 1.20	52.54 ± 0.87**
		K	33.19 ± 0.54	30.38 ± 0.44**	32.84 ± 1.10	36.27 ± 0.22**

[a] Values expressed as the mean ± standard error of the mean at the 0.05 (*) or 0.01 (**) level of confidence as determined by a *t* test. *N* = number of observations.

Table II. Effect of *in Vitro* DDT Treatment (30 min) on the Na^+,K^+-Dependent ATPase Activity in Liver (L), Intestinal Mucosa (I), Cloacal Bladder (B), and Kidney (K) Whole Homogenates from Five Turtle Species

			μg of phosphate/mg of protein per 30 min[a]			
				DDT concentration		
Species	*N*	Organ	Control	53 μM	5.3 μM	0.53 μM
Graptemys geographica	7	L	11.00 ± 0.63	9.54 ± 0.46*	0.08 ± 0.83**	2.51 ± 1.37**
		I	5.93 ± 0.63	5.10 ± 0.66	9.09 ± 1.43**	6.14 ± 1.31
		B	8.43 ± 2.80	7.61 ± 2.60	8.17 ± 0.92	5.00 ± 4.60
		K	23.06 ± 2.00	15.08 ± 2.31**	0.54 ± 5.42**	8.00 ± 2.66**
Chelydra serpentina	3	L	6.41 ± 0.10	3.79 ± 0.17**	1.84 ± 0.17**	2.87 ± 1.03*
		I	0.73 ± 1.34	0.00	0.00	4.00 ± 0.31**
		B	3.67 ± 0.90	1.48 ± 0.31**	4.45 ± 0.66	0.00
		K	7.05 ± 0.35	4.32 ± 0.12**	4.27 ± 0.14**	2.15 ± 0.81**
Pseudemys scripta	7	L	1.51 ± 0.34	4.54 ± 1.96**	4.32 ± 1.18**	0.18 ± 0.90
		I	4.75 ± 0.52	5.61 ± 0.55	9.39 ± 0.44**	3.94 ± 0.69
		B	0.18 ± 0.90	0.00	0.00	0.00
		K	7.09 ± 0.48	11.11 ± 0.32**	10.74 ± 0.32**	11.07 ± 0.17**
Trionyx spinifer	4	L	2.46 ± 0.37	0.00	0.00	0.00
		I	8.69 ± 0.31	3.15 ± 0.46**	1.48 ± 0.30**	0.98 ± 0.34**
		B	20.16 ± 0.37	15.73 ± 0.32**	19.86 ± 0.41	7.05 ± 0.35**
		K	6.76 ± 0.33	5.94 ± 0.51	2.74 ± 1.04**	1.30 ± 0.35**
Chrysemys picta	4	L	6.66 ± 1.35	0.00	0.00	0.00
		I	8.70 ± 0.39	0.00	0.00	0.00
		B	2.91 ± 0.48	0.29 ± 0.71**	0.00	0.00
		K	7.23 ± 0.47	0.00	5.11 ± 0.72*	3.48 ± 0.87**

[a] Values expressed as the mean ± standard error of the mean at the 0.05 (*) or 0.01 (**) level of confidence as determined by a *t* test. *N* = number of observations.

geographica (Table I). For all organs assayed except the kidney, the Mg^{2+}-dependent ATPase levels were consistently higher in *Graptemys geographica* than for the other species. The lowest Mg^{2+}-dependent ATPase activity of the five species studied was found in the liver, cloacal bladder, and kidney homogenate of *Chelydra serpentina*. The Mg^{2+}-dependent ATPase activity was significantly inhibited in all tissues assayed in *Graptemys geographica*, *Chelydra serpentina*, and *Pseudemys scripta* at 53 μM DDT (Table I). The Mg^{2+}-dependent ATPase was stimulated in the liver tissue of *Chrysemys picta* at 53 μM DDT, and the cloacal bladder and kidney tissues were inhibited at 53 μM DDT. The cloacal bladder of *Pseudemys scripta* exhibited considerably more Mg^{2+}-dependent ATPase activity than did the other tissues of this species.

Ouabain-sensitive (Na^+,K^+)-dependent ATPase activity was consistently lower than the corresponding Mg^{2+}-dependent ATPase (Table II). The highest (Na^+,K^+)-dependent ATPase activity was in kidney homogenates of *Graptemys geographica*. There was very little (Na^+,K^+)-dependent ATPase activity in the intestine of *Chelydra serpentina* and the cloacal bladder of *Pseudemys scripta* using a 5% homogenate. Treatment at 0.53 μM DDT stimulated activity in the intestine of *Chelydra serpentina*.

According to several theories of active transport, ATP is specifically required for the transport of ions against concentration gradients and across membranes. The mechanism of action of DDT upon this ATPase system may be due to the uncoupling of oxidative phosphorylation which

causes a decrease in the phosphorylation product—ATP. This reduction in available free phosphate would be directly proportional to the reduction in total ATP produced.

The amount of inhibition present in the cloacal bladder and intestinal mucosa of all species indicates that DDT may have a pronounced effect upon absorption of metabolites due to a lack of energy required for active transport across the cellular membrane. In this study there was marked inhibition of intestinal mucosa and cloacal bladder ATPase after *in vitro* treatment with 53 μM DDT. It is possible that treatment with equal concentrations of DDT *in vivo* would cause a serious inability to effectively utilize metabolites.

The action of the lowest concentration (0.53 μM) of DDT in the kidney of all species studied except *Pseudemys scripta* resulted in low remaining (Na^+,K^+)-dependent ATPase activity. Assuming that the (Na^+,K^+)-dependent ATPase activity in the turtle kidney is similar in function to that of other vertebrates, the inability to reabsorb electrolytes from the glomerular filtrate due to enzyme inhibition by DDT may cause serious electrolyte imbalances.

The observed action of DDT in all tissues assayed might be related to the ability of the compound to alter the cellular membrane configuration by binding with the fat portion of the membrane. Since ATPase is a structural part of the membrane, the active site of the enzyme would be altered. Movement of substances by active transport would be blocked. If the uptake of DDT by turtles in a natural environment reaches tissue concentrations equal to those found in this study, the resulting ATPase inhibition may be sufficient to impair organ function.

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Mutagenicity of Ethylene Chlorohydrin. A Degradation Product Present in Foodstuffs Exposed to Ethylene Oxide

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Ethylene chlorohydrin (2-chloroethanol) is mutagenic for *Salmonella typhimurium* in which microorganism it induces mutations of the base-substitution type. This chemical also preferentially inhibits the growth of DNA polymerase deficient bacteria which is taken to indicate an ability to react with the DNA of living cells. In view of the possible presence of ethylene chlorohydrin in foodstuffs exposed to ethylene oxide, the potential hazard of human exposure to this chemical requires examination.

Ethylene oxide (EO) is widely used as a fumigant to sterilize foodstuffs, textiles, medical instruments, and a variety of other objects (Merck Index, 1968; Fishbein, 1969). EO has wide applications in agriculture as a pesticide (Merck Index, 1968; Fishbein, 1969; Fishbein *et al.*, 1970) and in the tobacco industry to shorten the aging process and to reduce the nicotine content of tobacco leaves (see Fishbein, 1969; Fishbein *et al.*, 1970). The toxicity and mutagenic potential of EO are well recognized (Fishbein *et al.*, 1970); however, because of its volatile nature, it has generally been assumed that EO was safe, not leaving a residue (see, however, Fishbein, 1969; Fishbein *et al.*, 1970). It has been found, however, that under conditions for effective fumigation, EO reacted with moisture and chloride ions to form ethylene chlorohydrin (2-chloroethanol), a nonvolatile (bp 129°) toxic substance found in foodstuffs exposed to EO (Wesley *et al.*, 1965; Ragelis *et al.*, 1968; Fishbein, 1969). In the present report it is shown that ethylene chlorohydrin possesses mutagenic properties and that it is presumably able to react with the DNA of living cells. These results confirm and extend the earlier mention of the mutagenicity of ethylene chlorohydrin (Voogd *et al.*, 1972). Because of these findings and the strong positive correlation between mutagenic and carcinogenic potentials (Ames *et al.*, 1973a; Miller and Miller, 1971; Rosenkranz, 1973), it is suggested that the possible health hazards resulting from the presence of ethylene chlorohydrin in foodstuffs be evaluated and that fumigation procedures using EO which keep the level of ethylene chlorohydrin generated to a minimum be devised.

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DESCRIPTION AND RATIONALE OF THE ASSAYS

Mutagenesis Assay. The procedure for detecting revertants to histidine prototrophy was essentially that described by Ames (1971). The *Salmonella typhimurium* strains used (TA 1530, TA 1535, and TA 1538) were kindly provided by Dr. Bruce N. Ames. Strains TA 1530 and TA 1535 are essentially isogenic; they have a base-pair change in the histidine G gene and can be used to detect mutagens causing base-pair changes. In addition, both strains contain the uvr B mutation (Ames, 1971; Ames *et al.*, 1973b). However, strain TA 1535 differs from TA 1530 in that it is a deep-rough strain deficient in cell envelope li-