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Ref

Synergism among Oral Carcinogens[1]

IV. The Simultaneous Feeding of Four Tumorigens to Rats[2]

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Chronic feeding experiments have been conducted with individual pesticides. While a number of reports have been published on the antagonistic or potentiating effect of acutely or subacutely toxic doses of pesticides, practically no studies have been conducted that would throw light on the effects of the simultaneous ingestion of low doses of several of these compounds (Frawley *et al.*, 1957; Casida *et al.*, 1963; Casida, 1964). Our studies were initiated several years ago in order to determine the additive, antagonistic, or possible synergistic action (the syncarcinogenic action[3]) of Aramite, DDT, methoxychlor, and thiourea, when fed to rats for a period of two years (Radomski *et al.*, 1965). Our studies were primarily concerned with the incidence of liver tumors and the degree of anatomic change in this organ. The publications dealing with the liver tumorigenic effect of Aramite, DDT, methoxychlor, and thiourea were reviewed critically.

Aramite. Oser and Oser (1960), using their own colony of FDRL rats (Wistar strain) found that "Aramite is a liver carcinogen." A hepatic hyperplastic nodule was noted in one of 20 rats fed 500 ppm throughout the life span. In a similar group of 20 animals fed 1580 ppm, "a significant incidence of pathologic liver changes was observed, which, in two out of 21 rats, were described as malignant. At 5000 ppm the liver lesions in six of the 20 rats were diagnosed histopathologically as hepatomas or cholangiomas." In a second report by Oser and Oser (1962), the potential carcinogenicity of Aramite was reinvestigated. In this two-year feeding study they used a total of 1500 rats of three strains [FDRL, Sprague-Dawley (SD), and Carworth Farm-Nelson (CFN)], which were fed concentrations of 0, 100, 200, and 400 ppm. Gross changes in the liver were noted to increase with an increase of Aramite in the diet, as did elevations in weight of this organ. Tumors, at sites other than the liver, were principally in the mammary, subcutaneous, and gonadal regions. With the exception of the SD rats (in which there was a high degree

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[3] Term introduced by K. H. Bauer for the "Zusammenwirkung" of several carcinogenic factors (Schmähl, 1966).

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of mortality due to pulmonary lesions), survival for two years ranged from 34 to 64% of 300 FDRL rats, and from 22 to 48% of 300 CFN rats. In the group of 200 control rats, survival was 60%. A significant increase in the incidence and severity of hyperplastic liver nodules was apparent with an increase in dosage (100, 200, and 400 ppm) in both the FDRL and CFN rats, but only two hepatocellular carcinomas were observed in the FDRL group fed the highest dosage. Popper *et al.* (1960), in another study, came to similar conclusions.

TABLE 1

LIVER TUMORS IN RATS: SUMMARY OF LITERATURE

Dose fed for approx. two years (ppm)	Number of rats affected	Liver pathology	Reference
Aramite			
500	1 of 20	Hyperplastic nodule	Oser and Oser (1960)
1580	2 of 20	Malignant liver changes	
5000	6 of 20	Hepatomas, cholangiomas	
DDT			
200	1 of 24	Adenomas or low grade hepatic cell carcinomas	Fitzhugh and Nelson (1947)
600	2 of 23		
800	1 of 14		
Methoxychlor			
500	1	Poorly defined nodule	Nelson and Fitzhugh (1951)
2000	4	Hepatic cell adenomas	
Thiourea			
100	3 of 5	Hepatic cell adenomas	Fitzhugh and Nelson (1966)
250	4 of 8		
500	2 of 8		

DDT. Deichmann *et al.* (1950) incorporated commercial DDT into the diet of Sprague-Dawley rats in concentrations of 30 and 150 ppm. Significant histopathologic changes were not noted in the tissues of the animals surviving for two years with the exception of "hypertrophy and hyperplasia of the hepatic cells" in the rats fed 150 ppm.

Fitzhugh and Nelson (1947) fed commercial DDT to groups of 24 Osborne-Mendel strain rats in concentrations of 200, 400, 600, and 800 ppm. Because of the toxicity of DDT, mortality was high in the 400, 600, and 800 ppm groups. Considering all groups, survival for two years ranged from 0 to 50%. The outstanding gross anatomical change noted was a dose-related increase in the size of the liver. After 18 to 24 months of feeding, four rats demonstrated one or more small hepatic cell tumors (adenomas or low grade hepatic cell carcinomas), as follows: 1 of 24 rats fed 200 ppm, 0 in 14 rats fed 400 ppm, 2 in 23 rats fed 600 ppm, and 1 in 14 rats fed 800 ppm. Eleven other rats showed nodular adenomatoid hyperplasia. "Taken together, the 15 rats having either liver tumors or nodular adenomatoid hyperplasia are numerically enough to strongly suggest a distinct minimal tumorigenic tendency of DDT." There was

neither tumor nor adenomatoid hyperplasia at 100, 50, or 0 ppm. Retardation of growth rate was evident in female rats fed 400–800 ppm, and in male rats fed 800 ppm. Other gross changes included nodular adenomatoid hyperplasia, and in some instances focal necrosis of voluntary muscle. The microscopic lesions ranged from slight at 100 ppm, to marked at 800 ppm (Fitzhugh and Nelson, 1966).

Methoxychlor. Nelson and Fitzhugh (1951) fed methoxychlor for two years to six groups of 24 Osborne-Mendel strain rats in concentrations ranging from 10 to 2000 ppm. For the total group, survival for two years was approximately 52%. In the group fed 100 ppm, a few foci were noted in the liver of one rat. At the next higher dose (500 ppm) there was evidence of a 4-mm poorly defined nodule; and in the 2000 ppm group, there were four rats, each with a single liver tumor, plus a fifth rat with a poorly defined liver nodule. The tumors were identified as hepatic cell adenomas. They were "obviously nonmalignant." Most anatomic changes were found in the females, and all but one of the tumors or nodules were noted in rats surviving two years. No significant anatomic changes were noted in other organs. The body weight of these animals increased normally except for the 2000 ppm group, where "mild stunting" was noted.

Hodge *et al.* (1952) fed methoxychlor to groups of 50 rats in concentrations of 25, 200, and 1600 ppm. There was retardation in growth in the rats fed the highest dose, but they noted no histopathologic changes attributable to the administration of methoxychlor.

Thiourea. Fitzhugh and Nelson (1948) fed 100, 250, 500, 1000, 2500, 5000, and 10,000 ppm of thiourea with the diet to groups of 18 Osborne-Mendel strain rats. The feeding of 2500 ppm and higher doses "stunted the growth of rats," and all animals fed these doses died within 17 weeks. Histopathologic studies revealed hyperplasia of the thyroid gland. This ranged from very slight to marked in the groups fed from 500 to 10,000 ppm. Of 29 rats surviving the two-year experiment, 14 showed liver tumors (hepatic cell adenomas). "There was general correspondence between dosage level and tumor size and incidence." The authors concluded that thiourea "induces liver tumors, without liver cirrhosis, in a large percentage of cases at concentrations which may be below those producing hyperplasia of the thyroid gland."

"The numbers were . . . 1000 ppm, in 5 of 8 survivors; 500 ppm in 2 of 8 survivors; 250 ppm, in 4 of 8 survivors; 100 ppm, in 3 of 5 survivors" (Fitzhugh and Nelson, 1966).

No liver tumors were noted in the controls used here; however, it is of interest that the incidence of distinct spontaneous hepatic cell tumors of the type noted in the thiourea (and DDT) treated rats is seen in about 1% of the stock animals used by Fitzhugh and Nelson.

Aldrin. Davis and Fitzhugh (1962) fed for two years a group of 70 C_3HeB/Fe mice a diet supplemented with 10 ppm of aldrin. A similar experiment was conducted with 10 ppm of dieldrin. At 18 months there were 32 survivors in the aldrin, and 33 survivors in the dieldrin experiment. Gross and histopathologic changes in the animals dying or killed after 18 months included 35 adeno-

mas in the aldrin group and 36 in the dieldrin group. Also noted were enlarged mesentery lymph nodes. The authors concluded that the feeding of 10 ppm of aldrin or dieldrin significantly increases the incidence of histologically benign liver tumors.

Cleveland (1966), in a recently published study on aldrin and dieldrin, reported that aldrin and dieldrin when fed to rats in concentrations of 2.5, 12.5, and 25 ppm for a period of two years are neither tumorigenic nor carcinogenic.

EXPERIMENTAL

The dietary concentrations reported to be tumorigenic for the rat liver served as the basis for the pesticide feeding levels, namely: Aramite, 400 ppm (hyperplastic liver nodules); DDT, 400 ppm (hepatic cell tumors); methoxychlor, 2000 ppm (liver tumors); and thiourea, 100 ppm (liver tumors). Aldrin was added to our study when only mouse data were available; in this species, 10 ppm is tumorigenic.

Seven groups of 30 male and 30 female weanling Osborne-Mendel strain rats were placed on test diets. The animals were raised in our laboratory from a stock colony obtained from the FDA seven years ago. Each compound was added to ground Purina Laboratory Chow at a level of 50% of its tumorigenic dose. Thus, Aramite (technical grade, purity 90.6%) and DDT (recrystallized) were each fed at a concentration of 200 ppm, methoxychlor (recrystallized) at 1000 ppm, and thiourea (purity 100%) at 50 ppm. Aldrin (technical grade, purity 95%) was added to the basic diet at a level of 5 ppm. The sixth test group was fed a diet supplemented with a mixture of these pesticides, in a total tumorigenic dose of 200%, as follows: Aramite, 200 ppm; DDT, 200 ppm; methoxychlor, 1000 ppm; and thiourea, 50 ppm. This diet is referred to as mixture No. 1. The seventh group was fed the same diet as group 6, except that aldrin (5 ppm) replaced thiourea (mixture No. 2). The eighth group of 30 male and 30 female rats was fed an uncontaminated diet. All diets, as well as water, were supplied ad libitum.

The animals were housed in pairs. They were observed daily. Body weights were recorded once every month. Hematologic studies (hematocrit, hemoglobin, erythrocytes, and total and differential leukocytes) were carried out on 10 rats per group, picked at random from all groups except those fed thiourea and mixture No. 1, after 0, 3, 6, 12, 18, and 24–27 months of feeding. Animals found dead, and those sacrificed, were examined as promptly as possible for gross anatomical changes. The following organs were weighed: liver, lung, spleen, heart, brain, and kidney. All tumors, regardless of site, and the following organs were examined histologically: liver, heart, lung, brain hypophysis, kidney, spleen, pancreas, stomach, small intestine, and testes or ovaries. Feeding of the control diet and the diets supplemented with aldrin and mixture No. 2 was discontinued after 25 months. After 26 months, feeding of thiourea and mixture No. 2 was discontinued and the rats were sacrificed. Aramite, DDT, and methoxychlor diets were continued for 27 months.

RESULTS

Behavior and Growth

The general appearance of the rats in the seven experimental groups did not differ from the control group. After approximately 12 months of feeding, most of the rats fed the mixtures were hyperactive and demonstrated slight tremors. Signs of respiratory illness frequently seen in certain strains of rats were minimal. Compared to control groups used in other two-year rat feeding experiments in our laboratory, the controls in this study not only gained weight somewhat more rapidly during the first year, but maintained this weight gain until the feeding experiment was terminated at 24 months.

With the exception of the female rats fed methoxychlor and mixtures No. 1 and No. 2, and male rats fed mixture No. 1, the gain in body weight was normal. It was during the second year that the weights of the rats fed methoxychlor and the mixtures remained essentially stationary. The weights of the control and experimental animals fed the individual compounds did not differ significantly (except female rats fed methoxychlor). In spite of the subnormal gain in body weight of some of the rats, they appeared vigorous and healthy. This is also reflected in the rate of survival of the animals fed mixtures No. 1 and No. 2. In six of the eight groups, the survival rate of females exceeded that of males. After 24 months, the survival rate, based on 60 rats per group, was as follows: methoxychlor 40%, Aramite 45%, thiourea 51%, control 55%, aldrin 65%, DDT and mixture No. 2 66%, and mixture No. 1 70% (Table 2).

Organ Weights

The liver, kidney, brain, heart, spleen, lung, and gonads were weighed. Only the liver weight differed significantly from the controls (Table 3). The mean liver weights of male rats fed Aramite, DDT and mixture No. 2 were greater than those of the controls, while the mean liver weights of female rats fed methoxychlor and mixture No. 2 were less than the mean liver weights of the controls. Since we were concerned with the possible additive action of these compounds, it was not unexpected that the mean liver to body weight ratios of both male and female rats fed DDT, mixture No. 1 and mixture No. 2, and of males fed Aramite, were significantly greater, statistically, than the ratios for the control animals.

Hematology

The values for hematocrit, hemoglobin, erythrocytes, and total and differential leukocytes in the male and female experimental animals remained within normal ranges and did not differ from the values found in the control rats during the entire experiment.

Gross Pathology

As expected in rats of all groups, there were marked changes in the lungs with congestion, hemorrhage, pneumonitis, and some abscesses. The livers of some animals of all groups were slightly enlarged, congested, mottled or they

TABLE 2

MORTALITY OF RATS FED PESTICIDES[a]

Feeding time (months)	Control diet			Aldrin, 5 ppm			Aramite, 200 ppm			DDT, 200 ppm			Methoxychlor, 1000 ppm			Thiourea, 50 ppm			Mixture No. 1[b]			Mixture No. 2[c]		
	M	F	T	M	F	T	M	F	T	M	F	T	M	F	T	M	F	T	M	G	T	M	F	T
12	2	3	5	4	1	5	3	3	6	1	2	3	2	1	3	2	5	7	2	3	5	1	1	2
18	4	3	7	3	3	6	3	1	4	5	3	8	1	2	3	4	5	9	5	5	10	2	6	8
24	9	12	21	13	15	28	2	15	17	9	20	29	10	8	18	7	8	15	13	14	27	11	18	29
Killed or died, 25–27	15	12	27	10	11	21	22	11	33	15	5	20	17	19	36	17	12	29	10	8	18	16	5	21
Percent survival at 24 months	50	60	55	66	63	65	27	63	45	50	83	66	43	37	40	43	60	51	66	73	70	50	83	66

[a] Each group comprised 30 male (M) and 30 female (F) rats. T = total.

[b] Mixture No. 1: Aramite, 200 ppm; DDT, 200 ppm; methoxychlor, 1000 ppm; and thiourea, 50 ppm.

[c] Mixture No. 2: Aramite, 200 ppm; DDT, 200 ppm; methoxychlor, 1000 ppm; and aldrin, 5 ppm.

showed fatty changes. Rats fed Aramite and thiourea showed these changes most frequently. Some of the kidneys were enlarged, discolored, or congested. Again, the animals fed Aramite were primarily affected. Occasionally, the spleen of an animal was mottled. The gastroenteric tract of a few rats was congested. There were a few minor changes in other organs and tissues, such as the pituitary and lymph nodes.

TABLE 3

EFFECT OF FEEDING PESTICIDES ON THE LIVER WEIGHT AND LIVER TO BODY WEIGHT RATIO OF RATS[a]

Group	Liver weight (gram)	Liver to body weight ratio × 100
	Males	
Control	19.11 ± 4.24	4.16 ± 0.91
Aldrin	16.91 ± 2.91	3.82 ± 1.00
Aramite	22.10 ± 5.96[d]	4.85 ± 1.44[d]
DDT	24.80 ± 5.53[e]	5.36 ± 1.46[e]
Methoxychlor	19.53 ± 4.24	4.35 ± 0.98
Thiourea	16.75 ± 3.50	3.71 ± 1.06
Mixture No. 1[b]	18.20 ± 5.10	4.91 ± 1.05[e]
Mixture No. 2[c]	22.01 ± 5.64[d]	5.17 ± 1.47[e]
	Females	
Control	14.64 ± 3.28	4.01 ± 1.11
Aldrin	16.20 ± 4.04	4.39 ± 0.96
Aramite	12.83 ± 5.03	3.99 ± 1.20
DDT	16.27 ± 3.98	5.14 ± 1.09[e]
Methoxychlor	12.27 ± 4.15[d]	4.26 ± 0.95
Thiourea	13.35 ± 3.98	4.07 ± 1.08
Mixture No. 1[b]	14.76 ± 4.72	5.70 ± 1.31[e]
Mixture No. 2[c]	12.47 ± 3.47[d]	4.84 ± 1.24[d]

[a] Mean value ± standard deviation.
[b] Mixture No. 1: Aramite, 200 ppm; DDT, 200 ppm; methoxychlor, 1000 ppm; and thiourea, 50 ppm.
[c] Mixture No. 2: Aramite, 200 ppm; DDT, 200 ppm; methoxychlor, 1000 ppm; and aldrin, 5 ppm.
[d] $p = <0.05$
[e] $p = <0.01$

The summary presented in Table 4, as well as the anatomic changes reported here, demonstrate that gross and histopathologic changes in rats fed mixtures No. 1 and No. 2 were neither more frequent nor more severe than those noted in rats fed the pesticides individually. The most severe changes were noted in rats fed DDT.

Histopathologic Changes

A comparison of the histopathologic changes in the organs primarily affected in the animals fed the individual pesticides and the mixtures revealed the following:

Aldrin (5 ppm)

Liver—Moderate passive congestion and slight parenchymatous degeneration.

Lungs—Lymphopenia in lymph nodes around the larger bronchi. There was some bronchiolar stenosis, with papillary ingrowths where the epithelial lining cells were greatly swollen (containing eosinophilic cytoplasm and enlarged nuclei in which the chromatin was clumped).

TABLE 4

SUMMARY: INCIDENCE OF GROSS PATHOLOGY IN RATS FED PESTICIDES

Organ	Control	Aldrin	Aramite	DDT	Methoxychlor	Thiourea	Mixture No. 1	Mixture No. 2
Lung	50	38	55	52	58	42	58	38
Liver	6	7	10	6	7	13	7	5
Kidney	1	3	7	3	1	0	3	1
Spleen	1	1	2	4	2	3	3	2
Enteric tract	1	0	4	0	0	1	2	5
Lymph nodes	1	1	2	1	0	0	0	0
Pituitary	2	1	1	1	0	0	0	0
Brain	0	1	0	0	0	0	0	0

[a] The figures refer to the number of rats affected per group of 60.

Kidney—Slight infiltration of lymphocytes in the cortex, vascular congestion (inter- and intratubular) with some hyaline casts of hemoglobin in the tubules.

Spleen—Moderate to marked congestion in the red pulp with slight hemolysis, megakaryocytes, and an increased number of hemosiderin-laden macrophages.

Aramite (200 ppm)

Liver—Hydropic swelling, granular cytoplasm, slight fatty metamorphosis, small focal areas of centrolobular necrosis and passive congestion.

Lungs—Marked hyperplasia of the lymph nodes adjacent to the larger bronchi, with moderate lymphocytic cuffs and follicles about the blood vessels, and scanty mitosis.

Kidney—Slight infiltration of lymphocytes in the cortex, vascular congestion (inter- and intratubular) with some hyaline casts of hemoglobin in the tubules.

Spleen—Moderate to marked congestion in the red pulp with slight hemolysis, megakaryocytes, and an increased number of hemosiderin-laden macrophages.

DDT (200 ppm)

Liver—Diffuse centrolobular hypertrophy and some hyperplasia, with enlarged hyperchromic nuclei. Other areas with relatively large vesicular nuclei containing one to three large prominent basophilic nucleoli and some binu-

cleated cells. Margination cytoplasmic granules, fatty metamorphosis, and passive congestion. One rat exhibited a hepatoma, with many multinucleated cells, a foci of necrosis and fatty metamorphosis.

Lungs—Moderate lymphocytic cuffs and follicles about the blood vessels with scanty mitosis. One rat had a squamous bronchial metaplasia in a large bronchus.

Kidneys—Focal lymphocytic infiltration into the cortex, moderate to marked vascular congestion, a few small hemorrhages (inter- and intratubular), and some hyaline casts in the tubules. The tubules showed mild degeneration and some necrosis of the tubular epithelium, some of the cells exhibited polycystic degeneration; they were lined by a flattened epithelium.

Spleen—Moderate to marked congestion in the red pulp and slight hemolysis. There were some megakaryocytes and an increased number of hemosiderin-laden macrophages.

Methoxychlor (1000 ppm)

Liver—Hydropic swelling, granular cytoplasm, slight fatty metamorphosis, small focal areas of centrolobular necrosis and passive congestion.

Lungs—Moderate lymphocytic cuffs and follicles about the blood vessels with scanty mitosis.

Kidneys—Very slight infiltration of lymphocytes into the cortex, very slight congestion in the tubules.

Spleen—Moderate to marked congestion in the red pulp with slight hemolysis, megakaryocytes, and an increased number of hemosiderin-laden macrophages.

Thiourea (50 ppm)

Liver—Hydropic swelling, granular cytoplasm, slight fatty metamorphosis, small focal areas of centrolobular necrosis and passive congestion.

Lungs—Moderate lymphocytic cuffs and follicles about the blood vessels with scanty mitosis.

Kidney—Slight infiltration of lymphocytes in the cortex, very slight congestion in the tubules.

Spleen—Moderate to marked congestion in the red pulp with slight hemolysis, megakaryocytes, and an increased number of hemosiderin-laden macrophages. There were some giant follicles, lymphoreticular hyperplasia, small foci of myeloid metaplasia, as well as some endothelial and giant multinucleated cells and some atypical cells which could not be classified.

Mixture No. 1

Liver—Many focal areas of centrolobular hypertrophy, with large, slightly hyperchromatic nuclei, accompanied by marked fatty metamorphosis and lipospheres formed by proliferation of smooth-contoured endoplasmic reticulum. There were also focal areas of ischemic necrosis, some proliferation of bile ducts and ductules, cellular infiltration by lymphoreticular cells and also some eosinophils.

Lungs—Moderate lymphocytic cuffs and follicles about the blood vessels with scanty mitosis.

Kidney—Slight infiltration of lymphocytes in the cortex, vascular congestion (inter- and intratubular) with some hyaline casts in the tubules.

Spleen—Moderate to marked congestion in the red pulp with slight hemolysis, megakaryocytes, and an increased number of hemosiderin-laden macrophages.

Mixture No. 2

Liver—Spotty necrosis scattered through the parenchyma, fatty metamorphosis, fatty cysts, and slight portal inflammation. Some areas showed an irregular arrangement of hepatic cell plates, with circumscribed regeneration, including centrolobular hypertrophy and some binucleated cells.

Lungs—Slight hyperplasia of bronchiolar epithelium which was growing both in the terminal bronchiole and in the damaged alveoli, moderate lymphocytic cuffs and follicles about the blood vessels with scanty mitosis.

Kidney—Slight infiltration of lymphocytes in the cortex; vascular congestion (inter- and intratubular) with some hyaline casts in the tubules.

Spleen—Moderate to marked congestion in the red pulp with slight hemolysis, megakaryocytes, an increased number of hemosiderin-laden macrophages, some giant follicles, lymphoreticular hyperplasia, small foci of myeloid metaplasia, some endothelial and giant multinucleated cells, and some atypical cells that could not be classified.

Summary

The liver showed the most significant damage. In a decreasing order of severity, the groups ranked as follows: DDT, mixture No. 1, mixture No. 2, Aramite, methoxychlor, thiourea, and aldrin.

As expected, the lungs of most rats in all groups (including controls) showed moderate to marked vascular congestion, with hemorrhagic extravasation into the alveoli. The alveolar epithelial lining cells were swollen and desquamated, with degenerative changes, accompanied by slight proliferation or infiltration by septal cells and lymphocytes into the alveolar walls. These changes in the lungs were not the results of feeding the pesticides.

The kidneys of the rats fed DDT showed the most severe histopathologic changes. Aramite, mixture No. 1, mixture No. 2, thiourea, and aldrin induced only slight kidney changes, with methoxychlor having the least effect.

The spleen of some rats in all groups showed congestion, but the rats fed DDT, thiourea, and mixture No. 2, showed the largest number of unusual cells.

The hearts of some animals fed thiourea and mixture No. 2 (containing thiourea) showed small focal areas of interstitial myocarditis. This was probably due to secondary infection and not related to the feeding of these compounds.

All other organs and tissues were essentially normal.

Incidence of Tumors

As a rule, only one tumor per rat was found. The number of experimental rats so afflicted (all experimental groups including rats fed mixture No. 1, but not rats fed mixture No. 2) ranged from 10 to 17 per group. The total number of tumors per group was as follows: control, 15, one of these was malignant; aldrin, 15, two of these were malignant; Aramite, 16, two of these were malignant; DDT, 11, one of these was malignant; methoxychlor, 11, one of these was malignant; thiourea, 21, four of these were malignant; and mixture No. 1, 10, two of these were malignant. It is noted that the control rats fell well within the range of the experimental groups. The tumor rate in the rats fed mixture No. 2 was considerably below that of all other groups; only seven of these animals developed tumors; three of these were malignant. With very few exceptions, and as is common, tumors were noted only in rats which survived feeding periods exceeding 20 months (Table 5).

TABLE 5

NUMBER OF RATS AND NUMBER OF TUMORS PRODUCED

Compound(s) fed	Conc. of pesticide in diet (ppm)	Feeding time (months)	Number of rats with tumor(s)			Number of tumors produced		
			M	F	T	M	F	T
Control	—	25	1	13	14	1	14	15
Aldrin	5	25	2	13	15	2	13	15
Aramite	200	27	2	12	14	2	14	16
DDT	200	27	1	9	10	1	10	11
Methoxychlor	1000	27	5	6	11	5	6	11
Thiourea	50	26	2	15	17	2	19	21
Mixture No. 1	1450	26	3	7	10	3	7	10
Mixture No. 2	1405	25	2	4	6	2	5	7

[a] There were 30 male and 30 female rats per group.

Without reference to a particular diet, most of the tumors were benign fibroadenomas or adenofibromas of the mammary gland (in 68 rats). Other organs affected to a much lesser extent included: blood (malignant lymphosarcomas in seven rats), benign subcutaneous fibromas or lipomas (in 12 rats), skin, three rats (1 squamous papilloma, 1 adnexal cell adenoma, and 1 malignant squamous carcinoma), uterus, three rats (2 leiomyomas, and 1 fibroma), gastroenteric tract, two rats (1 leiomyoma, and 1 adenocarcinoma), lungs, two rats with bronchogenic carcinomas, urinary bladder, one rat with a mesenteric fibroma, and one rat with a malignant mesothelioma. These anatomic changes cannot be related to the compounds fed.

Only *two* liver tumors were noted: one a benign tumor (hemangioma) in a female rat fed 200 ppm of Aramite; the other, a malignant lymphosarcoma in a female rat fed mixture No. 1. A total of 296 rats survived for approximately 24 months. Of these, 101 showed 109 tumors, of which 16 were malignant (Table 6).

TABLE 6

ORGANS IN WHICH TUMORS WERE FOUND IN RATS SURVIVING 18 TO 27 MONTHS[a]

Site	Control		Aldrin		Aramite		DDT		Methoxychlor		Thiourea		Mixture No. 1		Mixture No. 2	
	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F
Mammary				1[b]								1[b]				
		12		10		12		6		6		13		5		3
Subcutis		1	1	1				2	3			1	2	1		
Lung																2[b]
Blood		1[b]	1		2[b]							2[b]	1[b]		1[b]	
Skin	1								1[b]		1					
Stomach				1[b]			1									
Liver						1		1						1[b]		
Miscellaneous						1		1[b]	1		1[b]	2			1	
Total nonmalignant	1	13	2	11		14	1	9	4	6	1	16	2	6	1	3
Total malignant		1		2	2			1	1		1	3	1	1	1	2

[a] In a few rats tumors were noted at two sites in one animal.

[b] Malignant.

DISCUSSION

In a previously reported investigation from our laboratory, 50 ppm each of Aramite, DDT, methoxychlor, and thiourea, was fed to a group of 50 male and 50 female Osborne-Mendel strain rats for 24 months in a diet of ground Purina Laboratory Chow. Theoretically, this diet contained a combined liver tumorigenic dose of pesticides equal to 78%. This diet had no effect on weight gain, food consumption, or rate of survival. There was, however, a somewhat higher incidence of malignant tumors (fibrosarcomas, carcinomas, or intrahepatic cholangiocarcinomas) in both male and female rats fed the experimental compounds, but the difference was not statistically significant ($P > 0.05$) (Radomski *et al.*, 1965).

In a second 24-month feeding experiment, the total tumorigenic dose of these compounds was increased to 124%. The same strain of rat, basic diet, feeding, weighing and autopsy procedures were used; however, the number of groups was increased to six (30 males and 30 females per group) and the concentration of each tumorigen was increased from 50 ppm to 80 ppm. Four of the individual diets were supplemented with either Aramite, DDT, methoxychlor, or thiourea. The fifth diet contained 80 ppm of each of these compounds, while the sixth was uncontaminated and served as control. As in the previous study, no effect, either of feeding of the mixture or of any of the individual compounds was noted on weight gain, longevity, or mortality. At 24 months, the mean mortality of all groups was 65% for the males, and 60% for the females. Fatalities among the rats fed the mixture of tumorigens were actually slightly lower than among the control animals. The tumor incidence was very similar among both experimental and control groups. Only one liver tumor was noted: in a female control rat. One rat fed Aramite and two rats fed thiourea showed diffuse and focal necrosis of the liver, while one rat fed the mixture had severe degeneration of the liver (Radomski *et al.*, 1965). This experiment confirmed the conclusions of the first study, but showed that ingestion of a mixture of Aramite, DDT, methoxychlor, and thiourea, in a total liver tumorigenic dose of 124%, does not result in an additive liver tumorigenic effect.

The third experiment reported here was conducted under the same basic conditions except that the compounds were fed in a total liver tumorigenic dose of 200%. Except for a subnormal gain in body weight and an increased degree of liver pathology in rats fed diets supplemented with DDT or mixtures No. 1 and No. 2, no significant effects were noted. Food consumption records were not kept; therefore, caloric restriction cannot be excluded as a factor for weight reduction. Tannenbaum (1959), reported a decrease in the incidence of lymphosarcoma, and a reduction in induced mammary carcinoma in rats fed a calorically restricted diet. Another effect of such a diet is adrenal hyperfunction. This has been shown to lead to increased glyconeogenesis, which, according to Boutwell *et al.* (quoted by Tannenbaum), may induce and explain the inhibiting effect of caloric restriction on tumor formation. In our rats, no increase in the size of adrenal glands was noted.

According to Tannenbaum, the liver stands out as a unique organ with regard

to the influence of nutrition on tumor formation. In the mouse, spontaneous hepatoma is inhibited by a diet low in protein content; while in the rat, such a diet will generally enhance liver tumors induced by azo dyes. In general, if the protein content of a diet varies within limits that do not greatly affect body growth or weight, then such a diet has little effect upon the growth of neoplasms.

While the body weights of females fed methoxychlor and mixtures No. 1 and No. 2, and males fed mixture No. 1, were subnormal, the animals on the whole appeared healthy and active (perhaps even more active); their fur was glossy and their survival rate exceeded considerably that of the control animals. We therefore wonder to what extent a possibly reduced food intake or increased physical activity might have been responsible for the reduction in body weight.

The studies reported in this communication substantiate our previous conclusions based on studies conducted with diets containing theoretical liver tumorigenic doses of 78 and 124%. In the present study there were histopathologic changes in the liver, lung, kidney, and spleen of rats fed these pesticides. The most marked changes were in the liver (except for the unrelated lung changes). The severity and incidence of histopathologic liver changes were not increased, were not very frequent, and in fact were less marked in rats fed mixture No. 1 and mixture No. 2 than in rats fed DDT only. The lack of "additive" toxicity could have been the result of stimulation of microsomal detoxifying mechanisms, since it is well known that certain chlorinated hydrocarbons augment the activity of these enzymes. The possibility also exists that these compounds, for reasons unknown, are antagonistic in their liver tumorigenic action.

From the data available, it has become evident that it is fallacious to accept, without proper testing, that two or more compounds, which when administered separately induce the same pharmacologic effect or the same type and degree of gross or histopathologic changes, *are additive in their specific effects when fed as a mixture* for two years. It is known, for instance, that the simultaneous administration of 2-acetylaminofluorene and 4′-methyl-4-dimethylaminoazobenzene, or 4-dimethylaminoazobenzene and 4′-methyl-4-dimethylaminoazobenzene results in a higher incidence of liver tumors than the total incidence obtained when the same level of *each* of the compounds of the pair is fed singly (MacDonald *et al.*, 1952). 20-Methylcholanthrene has been reported to act in the rat as a promoter of liver tumors initiated by dimethylaminoazobenzene (Odashima, 1959); however, when fed simultaneously to rats, the carcinogenic polycyclic hydrocarbons (Miller *et al.*, 1958), as well as some noncarcinogenic azo compounds (Crabtree, 1955), appear to inhibit, rather than potentiate, the production of hepatic tumors by aminoazo dyes and 2-acetylaminofluorene. Production of liver tumors in the rat by aminoazo dyes is also inhibited by 2,5-dimethylbenzimidazole and other benzimidazole derivatives (Clayton and Abbott, 1958), trypan blue (Fujita *et al.*, 1958), and ethionine (Cestari, 1958). The fact remains that specific effects of combinations of chemical compounds can hardly be predicted. In a recent publication, Schmähl (1966) came to the conclusion that one can expect a syncarcinogenic action only when two carcinogenic agents

i.e., 4-dimethylaminoazobenzene and diethylnitrosoamine, exert the same cytotrophic effect on the same target tissue, in this case the liver. We have no argument with this hypothesis. However, we believe that an additive, synergistic, or syncarcinogenic effect may be induced also through cytotrophic effects of two or more compounds in cells of related or even different systems. Through a cytotrophic mechanism, the opposite may occur when two compounds are administered; namely, a protective action, or at least a reduction in the expected effect (Deichmann *et al.*, 1965).

CONCLUSIONS

Albino rats were fed diets supplemented with Aramite (200 ppm), DDT (200 ppm), methoxychlor (1000 ppm), thiourea (50 ppm), and aldrin (5 ppm) for 24–27 months. These compounds were selected because they have produced hepatic cell adenomas or carcinomas in some rats, and it was decided to use the incidence of liver tumors as the primary criterion for additive action.

The materials were fed singly, *each* in a dose representing 50% of the compound's liver tumor inducing dose, and in combination (mixture No. 1) in a total theoretical tumorigenic dose of 200%, which included Aramite (200 ppm), DDT (200 ppm), methoxychlor (1000 ppm), and thiourea (50 ppm). In mixture No. 2, aldrin (5 ppm) replaced thiourea.

The following criteria were used to determine additive effects: signs of intoxication, body weight changes, hematology, rate of survival, organ weights, incidence of liver tumors, gross and histopathologic liver changes, and pathology in other organs.

No additive effects were noted when mixtures No. 1 and No. 2 were fed except for an increased degree of central nervous system stimulation, and a questionable decrease in growth rate.

Considering the increased period of survival of rats fed mixtures No. 1 and No. 2, and the lower number of liver tumors produced in these rats, one cannot help but wonder whether the feeding of these mixtures produced an antagonistic type of effect; an effect marked or specific enough to reduce the number of tumors below the level found in the control rats.

These results substantiate the earlier conclusions of studies conducted in this laboratory in which mixtures of Aramite, DDT, methoxychlor, and thiourea were fed to rats for two years in total theoretical tumorigenic doses of 78% and 124%.

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