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Synergism among Oral Carcinogens
I. Results of the Simultaneous Feeding of Four Tumorigens to Rats[1]

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Although the notion that carcinogens may augment one another has been tested frequently by skin painting or subcutaneous injection, few experiments designed to test for synergism or potentiation have been performed with carcinogens administered orally. MacDonald *et al.* (1952) found that the simultaneous administration of 2-acetylaminofluorene and 3′-methyl-4-dimethylaminoazobenzene or 4-dimethylaminoazobenzene and 4′-methyl-4-dimethylaminoazobenzene resulted in a higher incidence of liver tumors than the sum of the incidences obtained when the same levels of each of the compounds of the pair were fed singly. In another study, 20-methylcholanthrene appeared to act in rats as a promoter of liver tumors initiated by dimethylaminoazobenzene (Odashima, 1959). However, fed simultaneously, the carcinogenic polycyclic hydrocarbons (Miller *et al.*, 1958), as well as some noncarcinogenic azo compounds (Crabtree, 1955), appear to inhibit rather than to potentiate the production of hepatic tumors in rats by aminoazo dyes and 2-acetylaminofluorene. This inhibition has been explained on the basis of a stimulation of the microsomal detoxifying system by polynuclear hydrocarbons (Miller *et al.*, 1958). 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 *et al.*, 1958).

The combination of 2-acetylaminofluorene with *dl*-tryptophan, indole, or indoleacetic acid has been shown to produce bladder cancer in rats (Dunning *et al.*, 1950; Dunning and Curtis, 1958; Boyland *et al.*, 1954). A similar effect was not observed when *dl*-tryptophan was fed to rats in conjunction with benzidine or 2-naphthylamine (Boyland *et al.*, 1954).

The two experiments reported herein were designed to test for the presence of an additive or synergistic effect among compounds that exert a tumorigenic effect on the liver of rats after ingestion.

METHODS

Preliminary experiment. The first experiment was a preliminary one involving the following four compounds: thiourea, Aramite,[2] DDT, and methoxychlor. Since it was

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[2] Aramite is the trademark name of the United States Rubber Company for the miticide 2-(*p*-*tert*-butylphenoxy) isopropyl-2-chloroethyl sulfite.

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not intended that the compounds would be fed individually, a dosage was selected which would be well below the tumorigenic dosage of each of these compounds. A search of the literature revealed the following tumorigenic levels: Aramite produces liver tumors at 400 ppm in the diet, but not at 200 ppm (Oser and Oser, 1962); DDT produces liver tumors at 200–600 ppm in the diet, but not at 100 ppm (Fitzhugh and Nelson, 1947); methoxychlor produces liver tumors at 2000 ppm in the diet, but not at 500 ppm (Lehman, 1952); thiourea produces liver tumors at as low as 100 ppm in the diet (Fitzhugh and Nelson, 1947).

The experimental diet consisted of a ground laboratory chow[3] (supplemented to the extent of 1% with cod liver oil) to which was added 50 ppm each of Aramite, DDT, methoxychlor, and thiourea. With the exception of thiourea, which was added in a finely ground crystalline form, the compounds were dissolved in corn oil before being added to the diet. This diet was fed to a group of 50 male and 50 female rats of the Osborne-Mendel strain. A similar group of 50 males and 50 females were fed control diet without the addition of the above substances. Feeding was begun at weaning and continued for a period of two years. Food consumption was recorded, and animals were weighed monthly. Rats which became moribund were sacrificed. All rats which died or were sacrificed were examined for the presence of tumors. Tumors which were found were subjected to histopathologic examination.

Controlled experiment. This experiment was conducted in an attempt to resolve the equivocal results of the preliminary experiment. The same strain of rat, basal diet, and feeding, weighing, and autopsy procedures were used as in the preliminary experiment. In this experiment, however, the number of groups was increased to six consisting of 30 males and 30 females per group, and the concentration of tumorigen was increased from 50 ppm to 80 ppm. The compounds and concentrations fed were as follows: Group I, basal diet only (controls); group II, 80 ppm of Aramite; group III, 80 ppm of DDT; group IV, 80 ppm of methoxychlor; group V, 80 ppm of thiourea; group VI, 80 ppm each of Aramite, DDT, methoxychlor, and thiourea.

RESULTS

Preliminary Experiment

Feeding of the mixture of four tumorigens for 24 months had no effect on weight gain, food consumption, or survival. There was a somewhat higher incidence of malignant tumors in both males and females fed the mixture of the four compounds when compared to the controls (Table 1). This difference in tumor incidence was largely due to an increased occurrence of malignant liver tumors in the experimental rats, particularly the males. Although these differences were not statistically significant ($P > 0.05$), the results were believed to be suggestive because the substances fed were liver tumorigens.

The liver tumors were classified histologically as fibrosarcomas, carcinomas, or intrahepatic cholangiocarcinomas.

Controlled Experiment

In this experiment, as in the preceeding one, no effect of feeding either the mixture or any of the individual compounds was noted on longevity, mortality, food con-

[3] Purina Laboratory Chow, Ralston Purina Company, St. Louis, Missouri 63119.

TABLE 1

TUMOR INCIDENCE IN RATS FED A MIXTURE OF 50 PPM OF EACH OF ARAMITE, DDT, METHOXYCHLOR, AND THIOUREA FOR TWO YEARS

	Control diet			Experimental diet		
	Males	Females	Total	Males	Females	Total
Total number of rats	50	50	100	50	49	99
Rats with malignant tumors	7	8	15	13	13	26
Rats with benign tumors	1	10	11	0	5	5
Site of malignant tumors						
Liver	4	6	10	10	9	19
Lung	0	1	1	0	0	0
Uterus	—	1	1	—	3	3
Testis	0	—	0	1	—	1
Breast	—	0	0	1	0	1
Subcutis	0	0	0	1	0	1
Lymph node	1	0	1	2	0	2
Stomach	0	0	0	1	1	2
Bladder	1	0	1	0	0	0
Spleen	1	0	1	0	0	0
Total malignant tumors	7	8	15	16	13	29
Site of benign tumors						
Vagina	—	1	1	—	0	0
Uterus	—	1	1	—	3	3
Breast	—	8	8	—	5	5
Prostate	1	—	1	0	—	0
Total benign tumors	1	10	11	0	8	8

sumption, or weight gain. The cumulative mortality at the conclusion of 24 months of feeding was 65% for the males and 60% for the females. Mortality among the animals fed the mixture of tumorigens was actually slightly lower than among the control animals which received no tumorigens.

The primary pathologic change seen in these rats was chronic murine pneumonitis. This condition was observed to approximately the same extent in all six groups of rats. Organs and tissues in which tumors were found included the mammary gland, skin, subcutaneous tissue, liver, lung, intestines, lymph nodes, uterus, testis, adrenal and hypophyseal glands. The incidence of tumors is summarized in Table 2. Undifferentiated bronchogenis carcinoma was seen in eight of the rats fed 80 ppm of DDT. In addition, bronchogenic carcinoma was seen in three of the rats fed the mixture. Two lung carcinomas were seen in the control rats. Diffuse and focal necrosis of the liver was seen in one rat fed Aramite and two rats fed thiourea. One rat fed the mixture had severe liver degeneration. Leukemias of the lymphosarcomatous type were seen in two of the experimental rats (one rat fed methoxychlor and one rat fed the mixture). Testicular tumors (Leydig cell adenomas) were observed in one rat fed methoxychlor and in one fed thiourea. It seems probable that these were all fortuitous occurrences. In contrast to the preliminary experiment, only one liver tumor was found in the entire experiment, and that one was in a control rat. A large number of female rats of all six groups exhibited benign hyperplasia of the pituitary

TABLE 2

TUMOR INCIDENCE IN RATS FED 80 PPM OF ARAMITE, DDT, METHOXYCHLOR, AND THIOUREA, SINGLY AND IN COMBINATION FOR TWO YEARS

	Male rats by group[a]						Female rats by group[a]					
	I	II	III	IV	V	VI	I	II	III	IV	V	VI
Total number of rats	30	30	30	30	30	30	30	30	30	30	30	30
Rats with malignant tumors	3	2	4	0	0	4	6	3	6	5	1	4
Rats with benign tumors	0	1	1	2	1	1	6	8	5	6	10	6
Site of malignant tumors												
Liver	0	0	0	0	0	0	1	0	0	0	0	0
Lung	1	2	3	0	0	2	1	1	5	1	1	1
Intestines	0	0	0	0	0	0	1	0	0	0	0	0
Uterus	—	—	—	—	—	—	0	2	0	0	0	1
Breast	—	—	—	—	—	—	1	0	0	0	1	2
Pituitary	0	1	0	0	0	0	0	0	1	1	0	0
Subcutis	2	0	0	0	0	1	0	0	0	2	0	0
Adrenal	0	0	1	0	0	0	1	0	0	0	0	0
Leukemia	0	0	0	0	0	0	0	0	0	1	0	1
Miscellaneous	0	0	0	0	0	1[b]	1[b]	0	0	0	0	0
Total malignant tumors	3	3	4	0	0	4	6	3	6	5	2	5
Site of benign tumors												
Testis	0	0	0	1	1	0	—	—	—	—	—	—
Breast	—	—	—	—	—	—	6	6	4	5	9	6
Subcutis	0	0	0	1	1	1	0	0	0	1	0	0
Adrenal	0	0	0	0	0	0	0	1	0	0	1	1
Eye	0	0	1	0	0	0	0	1	0	0	0	0
Miscellaneous	0	1[c]	0	0	0	0	0	1[d]	1[e]	1[f]	0	0
Total benign tumors	0	1	1	2	2	1	6	9	5	7	10	7

[a] Group I is the control group II received Aramite; group III, DDT; group IV, methoxychlor; group V, thiourea; and group VI, the mixture of all four compounds.

[b] Carcinoma metastatic to lymph node.

[c] Osteoma.

[d] Uterus.

[e] Intestine.

[f] Pituitary.

gland. This is a common occurrence, in our experience, among laboratory rats surviving for two years, and was not more prevalent in any one of the six groups.

DISCUSSION

It seems clear from these experiments that at the levels tested, these compounds are not additive or synergistic in their production of liver tumors in rats. Although an additive or synergistic effect might conceivably have been produced at a higher dosage, it should be remembered that the dosages used in these experiments are far above the minute levels of these substances which might be encountered on crops or prepared foods.

Perhaps the failure to observe a synergistic effect in these experiments may be explained by the observation of Hart *et al.* (1963) that chlorinated hydrocarbons stimulate the microsomal detoxifying mechanism.

SUMMARY

A mixture of four substances, namely, Aramite, methoxychlor, DDT, and thiourea was fed to rats at a level of 50 ppm of each in the diet for a period of 24 months. This dietary concentration is considerably below the tumorigenic dosage of each of the individual compounds. Compared to the control rats, a somewhat increased incidence of liver tumors was observed in the males, and to a lesser extent the females, given the combination of the compounds. The incidences of tumors at other sites in the control and treated groups were not markedly different.

A second experiment was performed using a larger number of rats in which the same four compounds were fed individually as well as in combination, at a higher dietary level (80 ppm). In this experiment the incidence of tumors of all types in the rats fed the mixture of four tumorigens was no higher than that in the rats fed single tumorigens. No liver tumor was observed in any rat receiving any of these compounds. Thus, it would seem that these four compounds do not exert a synergistic or additive tumorigenic effect when fed to rats at the levels employed.

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