N-arylsulfenyl derivative of carbofuran (Black *et al.*, 1973b).

A study of the comparative metabolism of **6** in houseflies and mice is currently in progress.

Overall, the N-substituted biscarbamoyl sulfides presented in this study represent a promising group of carbamate derivatives which deserve further examination. Bisaldicarb sulfide (**14**) is an outstanding systemic insecticide in laboratory tests and is currently undergoing field tests.

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Effects of Phenoxyacetic Acids on Rat Liver Tissues

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No drastic damaging effect was noted when male Long-Evans rats were given 2,4-dichlorophenoxyacetic acid (2,4-D) or 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) equivalent to 2–5 g/kilogram body weight over a 4- to 7-week feeding period. Response to herbicide treatment was dependent on animal age and on the duration of chemical feeding. Based on equal amounts of either chemical fed, animals showed stronger responses to 2,4,5-T than to 2,4-D. Feeding of 2,4,5-T caused an increase in liver fresh weight and dry weight per 100 g of body weight, whereas 2,4-D had little or no effect on liver weight. Herbicide-induced enlargement of the liver was associated with increases in most of the major cellular components on a per liver basis. Glycogen content was 50 to 100% higher in both 2,4-D- and 2,4,5-T-treated rats than in controls. Livers from 2,4,5-T treated animals but not 2,4-D treated animals contained increased quantities of RNA and protein. Total DNA was increased only 10% or less by the treatments, while nuclear DNA content was 15–45% lower in livers from 2,4,5-T-treated animals than those from control and 2,4-D-treated animals. Isolated liver nuclei from both 2,4-D- and 2,4,5-T-fed rats were 20–30% more active in *in vitro* RNA synthesis than control nuclei.

2,4-Dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) are considered to be strong auxins. They stimulate plant growth (at low concentration) by inducing cell division and/or cell enlargement (Key *et al.*, 1966; West *et al.*, 1960). Auxin treatment results in enhanced *in vivo* synthesis of nucleic acid and protein, as well as increased *in vitro* nuclear or chromatin RNA polymerase activity, both in intact and detached plant systems (Chrispeels and Hanson, 1962; Matthysse and Phillips, 1969; O'Brien *et al.*, 1968a,b; West *et al.*, 1960). Commercially the auxins have been used as selective herbicides and as defoliants (Johnson, 1971; Klingman, 1949). The mode of action of 2,4-D and 2,4,5-T as plant hormones has recently been discussed in relation to nucleic acid regulation (Cherry, 1970).

Phenoxyacetic acids have been shown to cause a number of abnormal conditions in animals. Effects on growth rate, survival values, and individual organ weights depend on concentration, degree of purity, and method of administration. Hemorrhagic gastrointestinal tracts and several abnormal hematological parameters have also been observed (Emerson *et al.*, 1971; Hansen *et al.*, 1971; Johnson, 1971; Radeleff, 1964; Rowe and Hymas, 1954; Sparschu *et al.*, 1971a).

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Courtney *et al.* (1970) reported that 2,4,5-T was teratogenic, fetotoxic, and fetocidal in two strains of mice, and considerable concern has been expressed with regard to possible danger to humans. It has since been confirmed that the 2,4,5-T used in these studies was significantly contaminated with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, a manufactural impurity. It currently appears that most but not all of the fetotoxic, fetocidal, and teratogenic effects observed are due to this impurity and not to 2,4,5-T *per se* (Johnson, 1971; Sparschu *et al.*, 1971b). The present study is concerned with some general effects of auxin herbicides on rats, as well as some specific biochemical effects on the liver.

MATERIALS AND METHODS

Administration of the Herbicides. Unless otherwise stated, male Long-Evans rats (4-week-old or 7-week-old) were housed two per cage under a regulated cycle of 12 hr of light–12 hr of darkness. Animals were maintained on a diet of Wayne Lab Blox (Allied Mills, Inc., Chicago, Ill.), and were provided water *ad libitum*. Both 2,4-D and 2,4,5-T of the analytical standard grade (containing no 2,3,7,8-tetrachlorodibenzo-*p*-dioxin at a sensitivity of 0.05

ppm) were supplied separately through paired dietary feeding at a concentration of 2 mg/g of powdered food. (The Dow Chemical Company, Midland, Mich., supplied 2,4-D and 2,4,5-T. Chemical analysis of 2,4,5-T had shown that it contained no 2,3,7,8-tetrachlorodibenzo-*p*-dioxin at a sensitivity of 0.05 ppm.)

Preparation of Liver Homogenates. Control or herbicide-treated animals were weighed, stunned by a blow to the head, and then decapitated. Livers were removed and immediately placed in ice cold 0.25 *M* sucrose–TKM (TKM–0.05 *M* Tris-HCl, pH 7.5; 0.025 *M* KCl; 0.005 *M* $MgCl_2$). All subsequent steps were carried out at 0–4°. The combined livers from two to six animals were minced in a 1:1 ratio (w/v) of ice cold 0.25 *M* sucrose–TKM and homogenized in a Potter–Elvehjem homogenizer using a motor-driven Teflon pestle. After connective tissue was removed by forcing the homogenate through four layers of cheesecloth, part of the liver homogenate was used for nuclei isolation. The remaining homogenate was stored at −22° and later used to determine the total carbohydrate, nucleic acid, and protein content of the liver.

Determination of Carbohydrate, Nucleic Acid, and Protein Content of Liver Homogenates. Liver homogenates were sequentially extracted for carbohydrate, nucleic acid, and protein by the method of Shibko *et al.* (1967). Nucleic acid and protein were precipitated with perchloric acid (PCA) and collected by centrifugation. The resulting pellets were washed three times with 5% PCA by centrifugation and the resultant supernatants were added to the initial supernatant. Glycogen was precipitated from the combined supernatants by addition of 2 vol of cold 95% ethanol and collected by centrifugation.

Glycogen pellets were washed twice with 95% ethanol and hydrolyzed to glucose in 1.0 *N* HCl (100°, 2 hr). Both glycogen–glucose and reducing sugar contained in the supernatant after glycogen removal were estimated by the method of Nelson (1944).

The initial PCA precipitates were dissolved in 0.3 *N* NaOH and the RNA was hydrolyzed (37°, 3 hr). The RNA hydrolysate was acidified with 70% PCA and cleared by centrifugation. The resulting pellets were suspended in 1.5% PCA for DNA hydrolysis (90°, 30 min). The DNA hydrolysate was cleared by centrifugation after adding cold 70% PCA. The final pellet of protein and lipid was dissolved in 0.1 *N* NaOH and protein content was estimated by the method by Lowry *et al.* (1951), using bovine serum albumin as a standard. RNA and DNA were determined by the orcinol and diphenylamine reactions, respectively (Burton, 1956; Dische, 1955;), using yeast RNA and calf thymus DNA as standards.

Isolation of Nuclei from Liver Homogenates. Nuclei were isolated by the procedure of Blobel and Potter (1966). A mixture of 1:2 (v/v) liver homogenate to 2.3 *M* sucrose–TKM was layered over 5 ml of 2.3 *M* sucrose–TKM in centrifuge tubes. The tubes were centrifuged for 1.5 hr at 22,000 rpm using a Spinco SW-25.1 rotor. Supernatant material was removed by suction and the centrifuge tube walls were blotted dry. Nuclear pellets were taken up in 15 ml of 0.25 *M* sucrose–TKM and pelleted by centrifugation (750 × *g*, 5 min). Washed nuclei were suspended in 0.25 *M* sucrose–TKM and used to assay for RNA polymerase activity and to determine nuclear DNA content.

Assay of RNA Polymerase Activity. Isolated nuclei were used to assay endogenous liver RNA polymerase activity. The standard assay mixture contained: 60 μmol of Tris-HCl, pH 8.0; 2.0 μmol of $MgCl_2$; 0.5 μmol of $MnCl_2$; 1.0 μmol of dithiothreitol; 0.4 μmol each of GTP, CTP, and ATP; 0.02 μmol of UTP; 0.00057 μmol of ^{3}H-UTP (13.0 Ci/mmol); and 0.05 ml of nuclei representing 5–9 μg of DNA in a final volume of 0.25 ml. Assays were for 10 min at 37° and were terminated by addition of 1 ml of 10 m*M* $Na_4P_2O_7$ and 3 ml of cold 10% trichloroacetic acid (TCA). After being held on ice for 15 min, the acid-insoluble product was collected on glass fiber filters (Whatman GF/A) and washed with 5% TCA. Filters were dried under infrared light and transferred to counting vials. Radioactivity was determined by scintillation spectrometry at a counting efficiency of 25% for tritium.

RESULTS

Response of Rats to Exposure to 2,4-D or 2,4,5-T. Preliminary observations indicated that most rats tolerated both herbicides at dosages amounting to 2–5 g/kg body weight over a 28- to 49-day period. Rats showed a much stronger response to 2,4,5-T than 2,4-D, based on equivalent amounts of chemical fed. Two deaths and four cases of illness were recorded among 52 rats fed 2,4,5-T, while no deaths or cases of illness were found in a group of 19 rats fed 2,4-D. As a result of 2,4,5-T treatment, a few animals became weak and were extremely inactive prior to death. At this stage animals nearly always refused to eat food containing 2,4,5-T; however, 2,4-D-treated rats only occasionally showed a reduced appetite. Generally, animals that were treated with 2,4,5-T and those treated with 2,4-D were as active as, and consumed food equal to, control animals during the treatment period. The total dosage of 2,4,5-T causing death or illness was 225–750 mg/rat and was dependent on the age of the animal.

Ratio of Body Weight to Liver Weight after Herbicide Treatment. Table I indicates that feeding 2,4,5-T caused an increase in liver weight/100 g of body weight. Gains in body weight and liver weight over the treatment period were dependent on animal age. Furthermore, 2,4,5-T-treated animals had a higher ratio of liver weight/100 g of body weight (4.2 to 5.2) than the controls for all feeding periods. The values for control animals varied between 3.6 and 3.9, while the liver weights of 2,4-D-fed rats were much less, with values between 3.3 and 3.7.

Determination of Carbohydrate, Nucleic Acid, and Protein in Liver Homogenates Following Herbicide Treatment. Increases in liver weight observed by 2,4,5-T feeding resulted from both increased total water and increased total dry matter per liver since no change in dry weight percentage was observed following treatment. Cellular components were sequentially separated into glycogen, RNA, DNA, and protein and each component was compared as percentage of control (Table II). An increase in glycogen, RNA, and protein content was found to be associated with increases in liver weight resulting from 2,4,5-T treatment. Liver glycogen was increased by 2,4-D treatment, while small decreases in RNA and protein contents were observed. DNA was increased slightly by both herbicides.

Glycogen Metabolism Is Very Active in Young Growing Animals. Liver glycogen content was increased 50–100% in herbicide-treated rats after 35 days of treatment, while the sum of reducing sugar plus glycogen was only 15–30% higher in herbicide-treated rats than in controls (Table III). However, the reducing sugar content was the same in 2,4-D-treated and control animals and 15–25% higher in the case of 2,4,5-T treatment.

Comparison of Nuclear DNA and Total DNA in Liver Homogenates from Herbicide-Treated Rats. Table IV indicates changes in the relative amount of nuclear DNA isolated from livers of 4-week-old rats fed the herbicide for 35 days. The 2,4,5-T treatment decreased nuclear DNA per liver by 33% in comparison to control rats. In other experiments, feeding 2,4,5-T 4–5 weeks (at the same rate) resulted in a similar decrease in 7-week-old rats. In agreement with the data given in Table II, the data indicate a change in nuclear DNA even though total DNA of treated rats remains about the same as the control. A greater effect was noted by 2,4,5-T feeding than 2,4-D in this regard.

Effect of Herbicides on RNA Polymerase Activity of

Table I. The Ratio of Liver Weight to Body Weight of Rats after Treatment with Herbicides

Initial age, weeks	Length of treatment, days	Chemical fed, mg/week	Liver wt (g)/100 g of body wt		
			Control	2,4-D	2,4,5-T
4	49	130	3.7 (3.6–3.9)	3.5 (3.3–3.7)	5.0 (4.7–5.2)
7	28	220	3.7 (3.5–3.8)	3.2 (3.0–3.5)	4.3 (4.2–4.5)

The data are the mean values of three separate experiments using a total of 22 rats per treatment. Figures in parentheses represent the range in values obtained.

Table II. Determination of Glycogen, Nucleic Acid, and Protein Content of Livers from Herbicide-Treated Rats

	Content per whole liver, mg				
	Control	2,4-D	% control	2,4,5-T	% control
Glycogen	123 (106–131)	156 (152–160)	126	163 (124–202)	132
RNA	48 (41–56)	41 (32–50)	86	54 (47–61)	112
DNA	5.3 (5.0–5.6)	5.8 (5.7–5.8)	109	5.8 (5.6–5.9)	109
Protein	1124 (924–1324)	1087 (849–1364)	97	1442 (1360–1524)	128

Rats, 4 weeks of age, were fed 18.6 mg of 2,4-D or 2,4,5-T each day for a period of 35 days (total dose 650 mg/animal). The data given are mean values for two experiments using four animals per treatment in each experiment. Figures in parentheses indicate the range of values obtained.

Table III. Changes in Reducing Sugar and Glycogen Contents in Liver Homogenates from Herbicide-Treated Rats

	mg/whole liver/100 g of body wt		
	Control	2,4-D	2,4,5-T
Reducing sugar	239 (214–262)	212 (169–252)	299 (214–355)
Glycogen	62 (48–77)	139 (97–184)	111 (105–121)
Total	301 (262–339)	351 (266–436)	410 (318–476)
Animals per treatment (total in three experiments	8	8	10

Rats, 4 weeks of age, were treated with 18.6 mg of 2,4-D or 2,4,5-T each day for a period of 35 days (total dosage 650 mg/animal). The data given are the mean values of three experiments. Figures in parentheses show the range of values.

Table IV. Recovery of Nuclear DNA from Liver Homogenates after Herbicide Treatment

Treatment	DNA per whole liver		% total DNA recovered from nuclei
	Total, mg	Nuclear, mg	
Control	5.3 (5.0–5.6)	4.2 (3.9–4.5)	79
2,4-D	5.8 (5.7–5.8)	3.9 (3.8–3.9)	66
2,4,5-T	5.8 (5.6–5.9)	2.8 (2.8–2.8)	48

Four-week-old animals were fed 18.6 mg of 2,4-D or 2,4,5-T each day for a period of 35 days (total dose 650 mg/animal). The data represent two experiments using a total of ten animals per treatment. The figures in parentheses indicate the range of values obtained.

Isolated Liver Nuclei. Low concentrations of 2,4-D and 2,4,5-T greatly augment the synthesis of RNA in susceptible plants (Chrispeels and Hanson, 1962). *In vitro* studies indicate that the enhanced synthesis is related to a stimulation of RNA polymerase activity (O'Brien *et al.*, 1968a,b). Therefore, it was of interest to determine whether RNA polymerase activity associated with nuclei of rats was enhanced by *in vivo* herbicide treatments. Liver nuclei from 2,4-D or 2,4,5-T-treated rats were 20 to 30% more active (on a DNA basis) than control nuclei in incorporating ^{3}H-UMP into RNA (Table V), which indicates that the auxin herbicides do induce both additional transcription of RNA and translation of this or other RNA into protein (Table II). However, the magnitude of the stimulation in rats is much less than noted for susceptible plants.

DISCUSSION

After 2,4,5-T treatment, a higher ratio of liver to body weight was found (Table I). The enlargement of the liver has been suggested as a normal physiological response to the presence of a foreign agent. The liver is thought to respond to foreign substances by increasing the production of microsomal enzymes and certain other proteins (Goldberg, 1966). This may be the case for the 2,4,5-T induced increase in liver size, as well as for the increase in RNA and protein (Table II), although microsomal enzymes were not investigated in this study. The livers from 2,4-D-treated animals were always lighter in weight than control livers (Table I), had a decreased RNA content, and did not demonstrate any accumulation of protein. The fact that the responses to 2,4-D are quite different (except for glycogen accumulation) from those rats fed 2,4,5-T may indicate a differential metabolism of the two herbicides.

The one- to twofold increase in liver glycogen caused by 2,4-D and 2,4,5-T feeding cannot be the result of a differential food intake, as each group of animals received equal quantities of food daily. Since decapitation greatly activates liver phosphorylase (Hornbrook and Brody, 1963), it may be that 2,4-D and 2,4,5-T interfere with the degradation of liver glycogen. These data could equally well be

Table V. Effect of *In Vivo* Herbicide Treatment on *In Vitro* RNA Polymerase Activity of Rat Liver as Measured by Incorporation of ³H-UMP into RNA

	Incorporation of ³H-UMP into RNA	
	net cmp/100 μg of DNA/10 min × 10⁻³	% control
Control	56 (47–63)	100
2,4-D	73 (56–91)	131
2,4,5-T	67 (52–81)	120

Rats, 4 weeks of age, were treated daily with 18.6 mg of either herbicide for 35 days (total dose 650 mg/animal). The data are presented as the mean values for two experiments using a total of four animals for each treatment.

explained, however, as an overproduction of glycogen as a result of herbicide feeding. Glycogen accumulation was also demonstrated when herbicides were administered in drinking water (unpublished data).

2,4,5-T effects were more pronounced when animals were fed starting at 4 weeks of age rather than at 7 weeks of age. This type of differential response to auxins with age has also been noted in studies with *Drosophila* (Dävring and Sunner, 1971). Low dosages of 2,4,5-T affected early oogenesis and caused chromosomal aberrations, while adult flies were unaffected even at higher dosages. Moreover, Kimberg and Loeb (1971) demonstrated that incorporation of thymidine into nuclear DNA was considerably less sensitive to cortisone-induced inhibition in older rats than it was in younger growing animals.

The observed reduction in nuclear DNA found in 2,4,5-T-treated rat liver leads to several speculations. Because there is virtually a constant amount of total DNA per liver, it may be suggested that the newly synthesized or preexisting nuclear DNA has been altered in structure. This alteration may prevent the nuclear material from coming through sucrose gradients upon ultracentrifugation, resulting in a lower yield of nuclear DNA. This possibility is in good agreement with the observations presented in Table IV. Also, Croker (1953) has reported the formation of micronuclei in onion root tips by the herbicide. The results can also be explained as an inhibition of nuclear DNA synthesis; the excess supernatant DNA may be associated with the microsomal and/or the mitochondrial fractions (Bond *et al.,* 1969; Kimberg and Loeb, 1971). At present, the mechanism of the inhibition (or lowered recovery) of nuclear DNA on 2,4,5-T treatment remains unresolved.

In the present study the major effects of 2,4,5-T on rat liver were an accumulation of glycogen and a reduction (or lower recovery) of nuclear DNA. Recently an inhibition of liver DNA-polymerase activity has been demonstrated in cortisone-treated rats (Henderson and Loeb, 1971). Cortisol has been shown to induce liver glycogen accumulation in adrenalectomized rats (Chagoya *et al.,* 1971). It is, therefore, of interest to determine whether the auxin herbicides interact with endogenous animal hormones such as the glucocorticoids.

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