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THE TOXICITY OF DIAMINODIPHENOXYALKANES

BY

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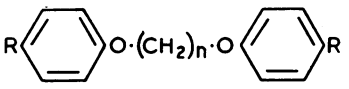
Representative members of a series of schistosomicidal diaminodiphenoxyalkanes were examined for their toxic effects in laboratory animals. Primary amino-derivatives were, in general, more toxic than secondary methylamino-compounds; tertiary compounds were less toxic than either. Large doses given by mouth or by injection to mice or rabbits produced intravascular haemolysis; the haemoglobin and erythrocyte counts began to increase again about a week after the dose. Mice which had been given large doses of diaminodiphenoxyalkanes developed symmetrical bald patches 2 to 3 weeks after treatment. The exposed skin appeared normal and new fur grew to cover the hairless areas in about 6 weeks. Large doses of drug delayed water diuresis in mice.

Many compounds of the series caused visual impairment when given to cats. In order to obtain a quantitative assessment of retinotoxic potency, a method was devised in which the ability of the retina of the frog to resynthesize rhodopsin was measured in treated and control animals. Compounds that produced blindness in cats also inhibited rhodopsin synthesis in frogs; the most toxic compounds were primary amines. Treatment with some tertiary amines caused retinal damage if the drugs were given for long periods. Diaminodiphenoxyalkanes may perhaps interfere in some way with the biological activity of vitamin A. However, toxic effects on the hair and retina were not prevented by supplements of synthetic or natural vitamin A.

The activity of a series of diphenoxyalkanes (I) against experimental schistosomiasis in mice has been reported by Standen and his colleagues (Raison and Standen, 1954, 1955; Caldwell and Standen, 1956; Standen and Walls, 1956).



Compounds of the same general type have been examined independently by Collins, Davis, and Hill (1954), and Edge, Mason, Wien, and Ashton (1956) have described the toxic effects of some members of the series upon the eyes of laboratory animals. The present paper deals with the toxic actions of representative members of the series tested by Standen.

A large number of compounds was studied, but for simplicity this account is concerned mainly with primary, secondary, and tertiary derivatives of diaminodiphenoxyheptane. The toxic actions of these compounds are typical of the series. The serial numbers and chemical structures of these compounds are: 153C51—R = NH_2; 413C52—R = NHMe; 27C53—R = NMe_2; 296C53—R = NEt_2; 252C53—R = $N(CH_2.CH_2OH)_2$.

METHODS

Diphenoxyalkanes.—Compounds were prepared and supplied as hydrochlorides by Drs. Caldwell, Raison, and Walls. Aqueous solutions became cloudy on dilution because of hydrolysis; solutions prepared for injection were freed from turbidity by adding a small amount of dilute HCl. Warming was necessary to dissolve some of the compounds with long chains. When large doses of drugs were given by mouth, suspensions were prepared with compound powder of tragacanth.

Toxicity to Mice.—Groups of 10 mice, weighing 20 to 25 g., were injected intravenously with 1% aqueous solutions of the drugs. Injections were given in 6 to 8 sec. Mortality was observed for three days; the LD50 of each compound was estimated graphically. Further experiments were made on groups of 10 or 20 mice which were given large doses (500 to 1,000 mg./kg.) of selected compounds by mouth on two or three successive days.

Effects on the Blood.—Haemoglobin determinations and red and white cell counts were made at intervals

during the treatment of mice, rabbits, and monkeys with compounds given by mouth, or by intravenous or subcutaneous injection. The method of Evelyn and Malloy (1938) was used to estimate methaemoglobin and sulphaemoglobin in whole blood. The serum was separated and examined spectroscopically.

Effect upon the Hair.—Loss of hair in mice caused by compounds of the series was assessed three weeks after treatment by awarding "scores" to individual mice and averaging the scores for groups of 10 or 20 animals. A score of 1 was awarded for partial loss, and 2 for complete loss of fur on head, back, or belly; a completely bald mouse would therefore score 6.

A number of supplements to the diet was given in attempts to antagonize the action upon the fur. Substances were incorporated in the diet, or were given by stomach tube or by subcutaneous injection, beginning before treatment with the diphenoxyalkane, and continuing throughout the experiment. Inositol, sodium *p*-aminobenzoate, calcium pantothenate, vitamin A (synthetic), fish liver oil concentrate, an alcoholic extract of liver (Armour's fraction 2), and various oils and fats were tested.

Effect upon the Excretion of Urine.—Groups of 10 mice were given doses of drugs by mouth; each animal received 0.5 ml. of solution. Urine was collected at intervals in a metabolism cage and the volumes excreted compared with those from mice given 0.5 ml. of water instead of drug.

Effect upon the Eyes of Cats.—Doses of selected compounds were given to cats by mouth or by subcutaneous injection. Acuity of vision was tested by the response of the cat to visual stimuli and by its behaviour when placed on a table or on the floor of a room. The fundi were examined with an ophthalmoscope, and histological preparations of some of the retinas were made.

Effect upon Rhodopsin Synthesis in Frogs.—The method used is related to one described by Zewi (1941) in a study of the actions of atropine and pilocarpine. Groups of 3 to 5 frogs (*Rana temporaria*) were given doses of selected compounds at 9 a.m., 5 p.m., and at 9 a.m. the following morning. The drugs were injected into the ventral lymph sac, or were given by stomach tube. The frogs were kept in about 1 in. depth of water in glass jars with perforated perspex lids. Immediately after the second dose the jars were placed in a white box so that the frogs were 20 in. below the central parts of two "warm white" fluorescent tubes (80 w.). The illumination at the bottom of each jar, measured with a light-meter, was 250 to 300 f.c. After being left for 17 hr. under the light, most of the retinal rhodopsin had been bleached. The jars were placed in complete darkness in a black box for 2 hr. so that resynthesis of rhodopsin could occur. The frogs were decapitated, the heads washed free from blood and the retinas removed from the eyes by the light of a dark-room lamp (Wratten, series 2). The retinas from each group of three frogs were added to 2.5 ml. of McIlvaine's phosphate buffer (*p*H 4.6) in a centrifuge tube and stirred to free the rods by inserting a stainless steel ball and moving the tube between the poles of a strong magnet. The tube was centrifuged for 20 min. in an M.S.E. angle centrifuge at 4,000 r.p.m., and the buffer discarded. The visual pigment was extracted from the rods with two portions of 1.5 ml. of 2% digitonin solution, the tubes being well stirred with the ball and centrifuged for 20 min. for each extraction. All these operations were performed in the dark. The combined extracts were made slightly alkaline by the addition of 0.2 ml. of saturated aqueous borax solution and transferred in the dark to the cell of a spectrophotometer for the measurement of absorption at 502 mμ. The extract was then bleached by exposure for 5 min. to the light of a 150 w. bulb at a distance of 15 cm., and the absorption measured again. The difference between the readings was proportional to the amount of light-sensitive pigment in the retinal extract. Control frogs which had been injected with 0.6% saline instead of drugs were used in each experiment, and the amount of rhodopsin resynthesized by treated frogs was expressed as a percentage of the control value. In a comparison of this kind it was considered unnecessary to go to further lengths to obtain the visual pigment pure.

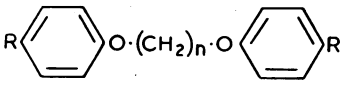
In some experiments, larger batches of 50 to 100 frogs were treated for longer periods and samples of three or five were taken from time to time to assess the effect of continued treatment and the rate of recovery when treatment ceased. Each group was exposed to standard conditions of light followed by darkness, alongside a group of normal controls treated with water or saline. A few experiments were made in which frogs were given large oral doses of synthetic or natural vitamin A (100,000 i.u./20 g. frog) before and during treatment with diphenoxyalkanes.

RESULTS

The acute toxicities to mice of a series of primary, secondary, and tertiary amino-derivatives are shown in Table I. The dose-response curves were almost parallel, and the figures in Table I

TABLE I

THE ACUTE INTRAVENOUS TOXICITY OF SOME DIPHENOXYALKANES TO MICE



Chain Length n	LD50 (mg./kg.)		
	R=NH$_2$	R=NHMe	R=N(Me)$_2$
5	140	180	120
6	100	71	46
7	71	220	250
8	110	56	72
9	110	240	84

have limits of error ($P=0.05$) of the order of 80 to 130%. Intravenous injection of large doses of primary amines caused death after a few minutes of convulsive movements. With the secondary and tertiary compounds convulsions were less marked, the main sign being flaccid paralysis of the hind limbs; respiration was not greatly affected in mice that did not die. The values of the LD50 of the primary amines tested were all of the same order. Secondary and tertiary amines with odd- and even-numbered carbon chains alternated in toxicity, those with 6 and 8 carbon atoms being more toxic than those with 5, 7, or 9. It is of interest that the schistosomicidal activity of tertiary methylamines also alternates, but in this instance compounds with even numbers of carbon atoms are a little less active than those with odd numbers (Raison and Standen, 1955).

When given by mouth, the drugs were tolerated by mice in single doses of 0.5 to 2 g./kg. When doses of 500 mg./kg. or more were given orally on 2 or 3 successive days, mice lost weight, the nose, paws, and tail became bluish in tint, and the fur was sometimes shed in symmetrical patches. Weight began to increase again about a week after treatment. Secondary amines were more prolonged in their action than the corresponding primary amines; the tertiary amines were less toxic than either when given by mouth.

Effect on the Blood.—The blue tint of the nose and paws of a mouse treated with large doses of diphenoxyalkanes was caused mainly by the presence of brownish-grey metabolites of the drugs in the blood and tissues. However, there was also a marked effect on the number of red cells, which decreased to about one-half the normal value after three oral doses of 500 mg./kg. The haemoglobin followed the erythrocyte count; both began to increase again at the end of a week. Fig. 1 shows the effect of heptane derivatives in the mouse and rabbit. Intravenous injection caused a very rapid decrease in red cells, and it was clear that the drugs, or their breakdown products, caused intravascular haemolysis. The fragility of the rabbit's erythrocytes was increased; the serum was stained reddish-brown and showed the absorption spectrum of haemoglobin. Small amounts of met- and sulphaemoglobin were present in the serum of treated mice and rabbits, but there was no significant change in serum bilirubin.

A short-lived polymorphonuclear leucocytosis followed the destruction of erythrocytes (Fig. 1). Blood films showed the presence of many immature red cells, and histological preparations made by Dr. D. Trevan from young rats which had

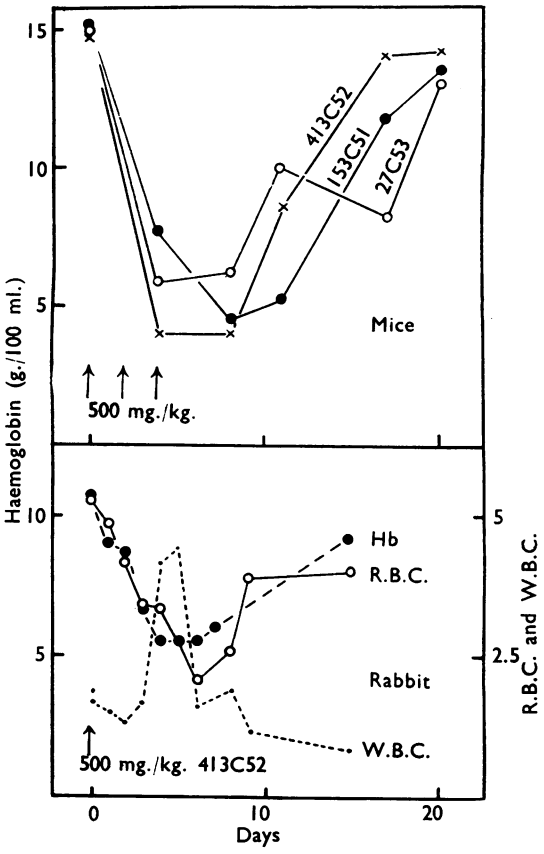


FIG. 1.—The effect of oral doses of diphenoxyalkanes on the blood of the mouse and rabbit. Ordinates for R.B.C. are in units of 10^6, and for W.B.C. in units of 10^4. Each point in the upper graph is the mean of two mice. The lower graph is from a rabbit weighing 4.3 kg.

been given oral doses of diphenoxyalkanes for 30 to 60 days showed that some of these new erythrocytes were derived from islands of erythropoietic tissue in the red pulp of the spleen. The bone marrow of most of the rats was normal.

Loss of Hair.—About 2 weeks after the administration of 2 or 3 large doses mice began to lose their hair. The remaining fur became silky and tousled; this change was also present in mice which were not sufficiently affected to show bald patches. A change of texture of coat occurred in rats, but no actual loss of hair was observed in rats, rabbits, cats, or monkeys. In mice, bare patches usually appeared on the head, back, and flanks, and the fur on the abdomen became sparse and fluffy (Fig. 2). Loss of hair was invariably symmetrical. The underlying skin was normal in appearance, and the claws and vibrissae were not noticeably affected. When "scores" were

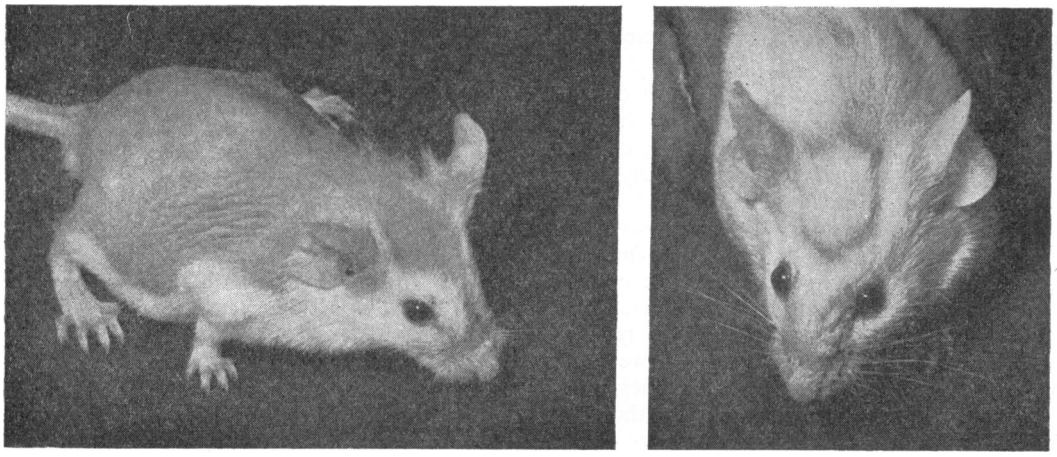


FIG. 2.—Examples of loss of hair in mice given 3 oral doses of 500 mg./kg. of a diphenoxyalkane 3 weeks previously. One mouse shows extensive loss of fur over the back and head; the other has a symmetrical pattern on the head only.

awarded to mice at the time of maximal effect (3 weeks after treatment), it was seen that the tertiary amines were the least, and the primary amines the most, toxic. In a typical experiment with the heptane derivatives the mean scores for groups of 10 mice were: 153C51—2.5; 413C52—1.7; 27C53—0.7. The ethyl compounds 296C53 and 252C53 were more toxic than the methyl compound 27C53. It is probable that the drug affected follicles which would have formed the coat in 3 weeks' time; these failed to develop, leaving bald patches when the older hairs had been shed. Subsequent crops of follicles were not affected, and 6 weeks after treatment the hair had grown again.

Phenolic compounds such as *p*-aminophenol and metol, which are possible metabolites of the diphenoxyalkanes, had no action of this kind on the fur of mice. Dietary deficiencies are a known cause of alopecia and therefore experiments were made in which supplements were given as possible antagonists to the action of 413C52. Inositol (500 mg./kg. daily) given by mouth or by subcutaneous injection, calcium pantothenate (500 mg./kg. daily), synthetic vitamin A acetate (1,000 i.u./mouse daily), or fish liver oil concentrate containing natural vitamin A (70,000 i.u./mouse daily) did not prevent loss of hair. Liver extract in enormous doses (20 to 200 mg./mouse daily) partially prevented loss of weight, but did not prevent loss of hair; sodium *p*-aminobenzoate (25 mg./kg. daily) increased the general toxicity of the drug and had no action on the loss of hair. Lard given at 10% in the diet prevented hair-loss in one experiment, but we were unable to repeat this in subsequent tests. No antagonistic action was shown by fats selected for their saturated or unsaturated fatty acid content (iodine values 8.8 to 86.1).

Inhibition of Diuresis.—Mice given large doses of primary, secondary, or tertiary amines by mouth excreted less urine than normal controls. Primary amines were the most, and tertiary amines the least, toxic in this respect (Fig. 3). Small doses of the primary amine 153C51 caused slight stimulation of the urine flow; doses of 75 mg./kg. or over caused inhibition. An effect on the urine flow was also observed when the dose was given by subcutaneous or intravenous injec-

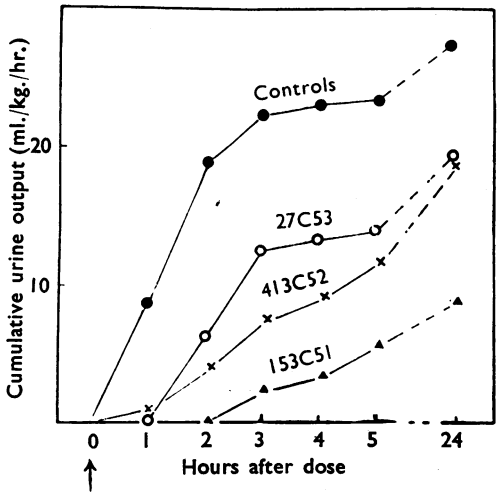


FIG. 3.—The effect of heptane derivatives upon the urine output of mice. Groups of 10 animals were given doses of 1 g./kg. orally. The curves show the means of two experiments.

tion. No significant histological changes were seen in the kidneys of animals which died after treatment with this drug.

Effects on the Eye.—Large and repeated doses of diphenoxyalkanes given by mouth had no apparent action upon the eyes of mice, rats, or guinea-pigs. On the other hand, many compounds when given orally had an effect on the eyes of cats. Within a few days the cat failed to respond to visual stimuli, refused to jump to the floor from a table, and explored the floor of the room with great care with its whiskers. The pupils were fully dilated, and failed to react to light. Examination of the fundus showed narrowing of the blood vessels and sometimes pallor of the optic disc. Pigmentation of the kind described by Edge *et al.* (1956) was not observed.

Sections of retinas showed areas of damaged pigment epithelium and affected rods. Many of the cells of the pigment epithelium were swollen and the pigment granules were distributed throughout the cytoplasm instead of being arranged in an orderly manner at the inner borders of the cells. In some areas the pigment in the epithelium was absent. Other layers of the retina were normal in appearance. The optic nerves were also examined and no abnormality was found.

Toxic effects on the retina were easy to obtain with primary and secondary amines; more prolonged treatment with large doses was necessary to get effects from tertiary methylamines such as 27C53. No blindness was produced by prolonged treatment with 296C53 or 252C53 by mouth.

The extent of blindness in cats is not easy to evaluate, and the experiments on frogs were designed to give a more objective measurement of the degree of toxicity of a drug to the retina. The results agreed very well with those obtained in cats.

TABLE II

RHODOPSIN IN THE RETINAS OF FROGS INJECTED WITH 153C51 AND EXPOSED TO PERIODS OF LIGHT AND DARKNESS

Groups of 3 frogs were given doses of 100 mg./kg. of 153C51 into the ventral lymph sacs at the beginning of the 24 hr. in the dark. The dose was repeated after 8 hr., and groups C and D were given a third dose after 24 hr. in the dark. Control groups were injected with 0.6% saline. Each rhodopsin figure was obtained by measuring the absorption of the retinal extract at 502 mμ before and after bleaching by light. The difference between the spectrophotometer readings is proportional to the amount of light-sensitive pigment in the extract. Resynthesis in the treated frogs was impaired.

Exposure to Light and Darkness	Rhodopsin per Frog	
	Treated	Controls
A. Dark 24 hr.	25	27
B. ,, 24 hr.; light 1 hr.	6	11
C. ,, 24 hr.; ,, 1 hr.; dark 1 hr.	12	42
D. ,, 26 hr.	37	57

Table II shows that exposure to light for only 1 hr. was effective in bleaching the greater part of the rhodopsin in the frog retina, and that a subsequent period of 1 hr. in the dark was sufficient for a considerable degree of resynthesis to take place. Frogs treated with 153C51 but kept in the dark retained most of their rhodopsin, and this was bleached in the normal manner when the animals were exposed to light (Table II). The drug prevented resynthesis when the animals were again put in the dark, and the effect was proportional to the dose (Fig. 4). Frogs which had been handled always gave higher rhodopsin figures

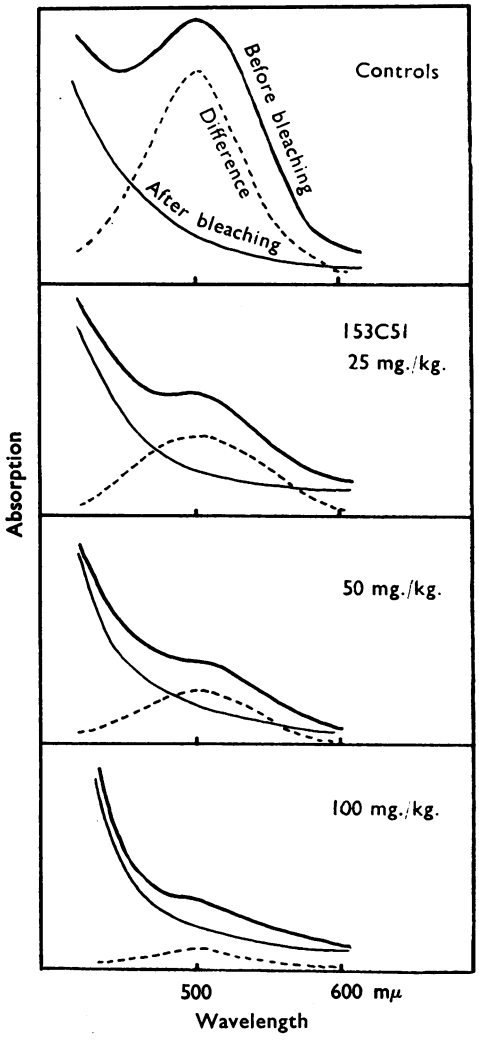


FIG. 4.—The effect of graded doses of 153C51 on the synthesis of rhodopsin in the frog retina. Groups of 3 frogs were used at each dose.

than frogs which had been left undisturbed; it was important therefore to give doses of frog-saline to control animals whenever doses of drugs were given to test groups. The dose-response curves obtained with the heptane derivatives are shown in Fig. 5. Doses given by mouth produced similar, but smaller, effects. The primary amines

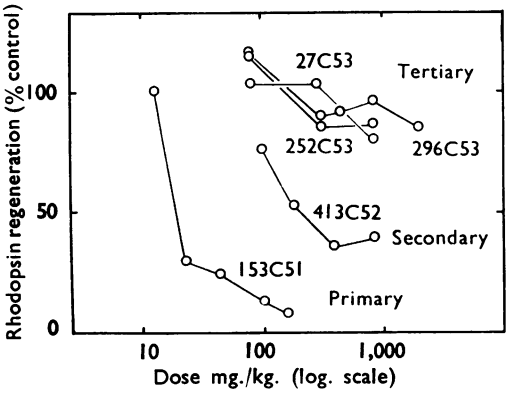


FIG. 5.—Dose response curves showing the relative toxicities of heptane derivatives to rhodopsin synthesis in the frog retina. Doses were given into the ventral lymph sac, and are calculated in terms of base. Each curve is the mean of several experiments.

were the most, and the tertiary the least, toxic. The effect of one course of 3 injections of 100 mg./kg. of 153C51 was sufficient to depress the rhodopsin extractable from the retinas of frogs exposed to standard light followed by dark conditions for 2 to 3 weeks. There was then a gradual return towards normal, but only 40% or less of normal ability to resynthesize rhodopsin was recovered 4 weeks after the drug was given. Some of the tertiary amines, such as 27C53, 296C53, and 252C53, had little or no action when given in the standard course of 3 doses. When large doses were given orally to frogs on 5 days a week for several weeks, the dimethyl-compound 27C53 produced toxic effects after 1 week and the diethyl-compound 296C53 showed an effect after 3 weeks (Fig. 6). The effect of the dihydroxyethyl-compound 252C53 was very slight.

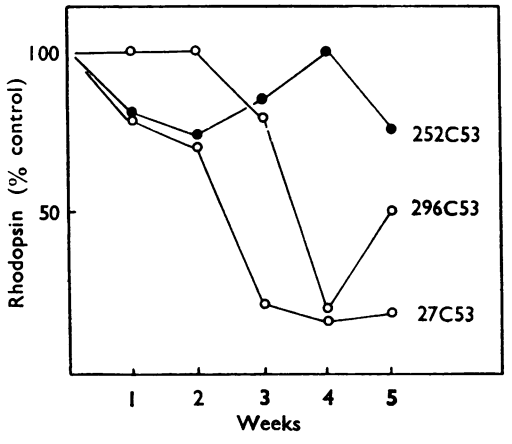


FIG. 6.—The effect of prolonged oral treatment with heptane derivatives on the rhodopsin content of frog retinas. Each drug was given daily for 5 days a week in doses of 200 mg./kg. Groups of 3 frogs were killed weekly and compared with a parallel group of frogs given 0.6% saline.

Large doses of natural or synthetic vitamin A given orally to frogs did not antagonize the retinotoxic effect of 153C51.

DISCUSSION

It is clear that an estimate of the toxicity of diphenoxyalkanes based upon intravenous LD50 values for mice would be misleading. From Table I it might appear that some of the primary amines are less toxic than secondary or tertiary derivatives. Nevertheless, the more important effect on the retina is most pronounced with primary amines. The effect on the retina is one of the most interesting aspects of the toxicity of diphenoxyalkanes. Carnivores and primates appear to be more sensitive than rodents to the retinotoxic action of oral doses of the drugs. In addition, it has been found by Dr. J. Newsome (private communication) that the tertiary amino-derivatives 296C53 and 252C53, which have low toxicity to the eye and powerful schistosomicidal activity in mice, have no detectable activity on *S. mansoni* infection in a baboon. These findings suggest that the drug itself, or secondary and primary amines derived from it, may be the retinotoxic agents, and that mice, guinea-pigs, and rabbits are capable of metabolizing diaminodiphenoxyalkanes given orally into substances which kill schistosomes but have minimal effects on the eye. A wide survey of the series has shown that retinotoxic activity in the frog and cat does not run parallel to schistosomicidal potency in the mouse, and it is possible that a member of the series will eventually be found which shows high schistosomicidal activity in primates without dangerous side-effects on the retina. Compounds which depart from the general structure in having amino-groups in the *m-* or *o-*, instead of the *p-*, position, and those with hydroxyl-groups substituted in the benzene rings, have neither retinotoxic nor schistosomicidal activity.

It is tempting to suggest that some of the toxic actions of the drugs may be brought about by interference with the biological activity of vitamin A. However, attempts to antagonize the

effects of the drugs on the hair and the retina with large doses of preparations containing synthetic or natural vitamin A met with no success. There is some evidence that the drugs are inhibitors of alcohol dehydrogenase, and this may be one way in which they could interfere with the synthesis of rhodopsin in the retina. Alcohol dehydrogenase catalyses the formation of the aldehyde retinene from vitamin A alcohol; the retinene then combines with retinal protein to form rhodopsin (Collins, 1954). An understanding of the mode of action of compounds of this series depends on a fuller investigation of their metabolism and of the effects of the drugs and their metabolites on biochemical processes.

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