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EFFECTS OF A RETINOTOXIC PHENOXYALKANE ON THE VISUAL CYCLE IN RABBIT RETINAE

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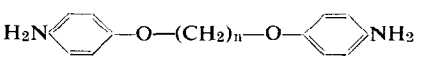
Abstract—Intravenous injection at a dose of 5 mg/kg into rabbits of the schistosomicidal and retinotoxic drug, 1:5-di-(*p*-aminophenoxy)pentane was shown to affect biochemical mechanisms associated with the visual cycle. One hr after injection re-synthesis of visual pigment in dark-adapted animals was inhibited about 50 per cent. In addition, in the dark, the drug caused an apparently spontaneous breakdown of about 16 per cent of visual pigment in the retina. Retinal alcohol dehydrogenase was inhibited about 42 per cent 1 hr after injection and the activity of the metabolically coupled hexose monophosphate shunt was also depressed.

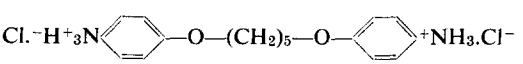
A SERIES of symmetrical diaminodiphenoxyalkanes having the general structure:—



were shown by Collins *et al.*[1] and by Raison and Standen[2] to possess a high degree of schistosomicidal activity.

The primary amines and monomethylamine derivatives showed a peak of activity at a chain length of $n = 7$–8. In general, the compounds showed decreasing activity as the size of the alkyl chain increased.

One of this series of compounds, viz.: 1:5-di(*p*-aminophenoxy)-pentane dihydrochloride



although being very active against *S. mansoni* in mice, unfortunately possessed marked toxic effects on the retinae of monkeys, cats and dogs as shown by Edge *et al.*[3]

Sorsby and Nakajima[4] were able to produce retinal damage in the rabbit following a single intravenous injection of a number of diaminodiphenoxyalkane drugs, the primary amine type being the most toxic. Amongst these drugs, 1:5-di-(*p*-aminophenoxy)pentane when given intravenously to rabbits at a dose of 60 mg/kg produced marked retinal damage involving the visual cell layer and eventually, the pigment epithelium. These changes were observable by direct ophthalmoscopy within 2–3 days and full degeneration was apparent within 21 days. Histologically, the visual cells and the pigment epithelium were affected most.

Ashton[5] described the retinal changes produced by 1:5-di-(*p*-aminophenoxy)pentane as being similar to those following the intravenous injection of sodium iodate and

consisting of destruction of the visual cell layer, with localized displacement of the outer nuclear layer together with marked degeneration and proliferation of the pigment epithelium.

Arden and Fojas[6] examined the effects of the diaminodiphenoxypentanes on the ERG of the rabbit. They found that the primary amines affected the amplitude and waveform of the dark-adapted ERG and reduced rate of dark adaptation. These authors argued that the results accorded with an alteration in rate of regeneration of visual purple.

Previous biochemical experiments (Reading and Davies, unpublished) had shown no marked or specific effect on enzymes of carbohydrate metabolism in the mammalian retina by the retinotoxic diaminodiphenoxyalkanes and in addition, Goodwin *et al.*[7] had found that these drugs prevented the resynthesis of visual pigment in amphibia.

For these reasons, it was decided to investigate the effect of 1:5-di-(*p*-aminophenoxy)pentane on enzymatic pathways associated with the visual cycle. These included the hexose monophosphate shunt (HMP) of direct oxidation of glucose and retinal alcohol dehydrogenase (retinene reductase). The HMP shunt pathway has been implicated in the normal functioning of the visual cycle in mammalian retina by the researches of Futterman.[8] After bleaching, reduction of retinene to retinol (vitamin A alcohol) by retinal alcohol dehydrogenase is metabolically coupled to the HMP shunt by the latter utilizing the NADP produced from the enzymatic reduction. The net result of bleaching of visual pigment is a stimulation of HMP shunt activity.

In addition, any direct effects on the stability of the visual pigment following injection of 1:5-di-(*p*-aminophenoxy)pentane were investigated.

METHODS

All reagents used were of Analar grade and specifically labelled radioactive glucose was obtained from the Radiochemical Centre, Amersham.

In all experiments, solutions of drug for injection were made up in physiological saline. Injections were made intravenously into an ear vein of the rabbit and the dose was 5 mg/kg. Dutch rabbits of about 1·5 kg were used throughout. The volume of solution injected was approximately 1·5–2·0 ml. In control experiments, rabbits were injected intravenously with 2·0 ml physiological saline.

Evaluation of hexose monophosphate shunt activity

Evaluation of the relative activity of the HMP shunt of glucose oxidation following a single intravenous dose of 5 mg/kg of 1:5-di-(*p*-aminophenoxy)pentane was carried out as previously described.[9] In this method, 25 mg quantities of retinal tissue were incubated at 37° in 2 ml Krebs–Ringer bicarbonate medium[10] containing 2 μc of either 1-^{14}C-glucose or 6-^{14}C-glucose diluted with inert carrier glucose to give a final concentration of 0·1% in the medium. The gas phase was 95% oxygen, 5% carbon dioxide. After 1 hr incubation, reaction was stopped by injecting acid into the medium, which also served to drive off dissolved carbon dioxide and ensured complete collection of carbon dioxide on filter papers soaked with alkali (also by injection).

The activity of the HMP shunt in the retina following drug administration was assayed by expressing the yields of $^{14}CO_2$ (called specific yields) in terms of the corresponding glucose substrate utilized. Thus, the difficulties encountered by expressing $^{14}CO_2$ yields alone, without reference to the amount of substrate utilized

in parallel experiments was overcome. Comparison of means of evaluation of HMP shunt activity in tissues were fully discussed by Katz and Wood[11] and the present method employs their suggestions.

Determination of retinal alcohol dehydrogenase (retinene reductase) activity

The effect of a single intravenous injection of 5 mg/kg of 1:5-di-(*p*-aminophenoxy) pentane on retinal alcohol dehydrogenase was estimated by determination of enzymatic activity in 0·25 M sucrose homogenates (two retinae to 1·0 ml sucrose) of excised rabbit retinae. Retinae were removed from enucleated eyes as rapidly as possible under ice-cold physiological saline, surplus liquid was removed, and the tissues were rapidly weighed on a torsion balance before adding to 0·25 M sucrose solution in the homogeniser tube. Homogenisation was made using a "Teflon" pestle. Details of the incubation mixture and spectrophotometric estimation of retinene production have been reported elsewhere.[12]

The method depends on the rate of production of retinene from the substrate vitamin A alcohol, the former being estimated by the thiobarbituric acid method.[13]

Evaluation of the effect of 1:5-*di*-(p-*aminophenoxy*)*alkane on the stability of the visual pigment*

Determinations of the visual pigment content of rabbit retinae following the intravenous injection of 5 mg/kg 1:5-di-(*p*-aminophenoxy) pentane were carried out by estimation of the retinene content of digitonin extracts of retinal tissue made after light or dark adaptation as described by Futterman and Saslaw.[13] For the dark adaptation experiments, the rabbits were kept in a completely dark room for 1 hr before any injections or estimations were made. Light adaptation (bleaching) was achieved by keeping the animals illuminated by four "photoflood" lamps, one at each side of the animal cage, placed about three feet from the cage centre.

In the dark adaptation experiments, all manipulations of the retinae and the injection of drug, were carried out in the light of a red (orthochromatic) darkroom safelight (Wratten Series 2).

RESULTS

Hexose monophosphate shunt activity

The results of the determinations of retinal HMP shunt activity following a single intravenous dose of 5 mg/kg of 1:5-di-(*p*-aminophenoxy)pentane are shown in Table 1. Although there was an initial indication of a slight stimulation of the HMP shunt at 7 hr after injection, the general tendency was towards an overall decrease in HMP shunt activity. All values, however, showed a high degree of variation.

Activity of retinal alcohol dehydrogenase (retinene reductase)

Results of determinations of retinal alcohol dehydrogenase activity carried out over the period, 1 hr to 7 days, after a single intravenous injection of 5 mg/kg of 1:5-di-(*p*-aminophenoxy)pentane are shown graphically in Fig. 1. The injection of the drug produced a marked and rapid effect on retinal alcohol dehydrogenase activity. One hr after injection enzyme activity was inhibited about 42 per cent, with a return to normal levels after 24 hr.

TABLE 1. HMP SHUNT ACTIVITY IN RABBIT RETINAE FOLLOWING INTRAVENOUS INJECTION OF 1:5-DI-(*p*-AMINOPHENOXY)PENTANE DIHYDROCHLORIDE (5 mg/kg)

Time after injection	Specific yield $^{14}CO_2$ $\frac{1\text{-}^{14}C \text{ glucose}}{6\text{-}^{14}C \text{ glucose}}$	% change in HMP shunt in retina
Control (no drug)	1·51 (± 0·21)	—
7 hr	1·86 (± 0·74)	15·0% (± 48·0%)
16 hr	0·80 (± 0·70)	−48·0% (± 45·0%)
24 hr	1·00 (± 0·41)	−36·0% (± 26·0%)
7 days	0·82 (± 0·24)	−47% (± 15·0%)

(S.E.M. in parentheses.)

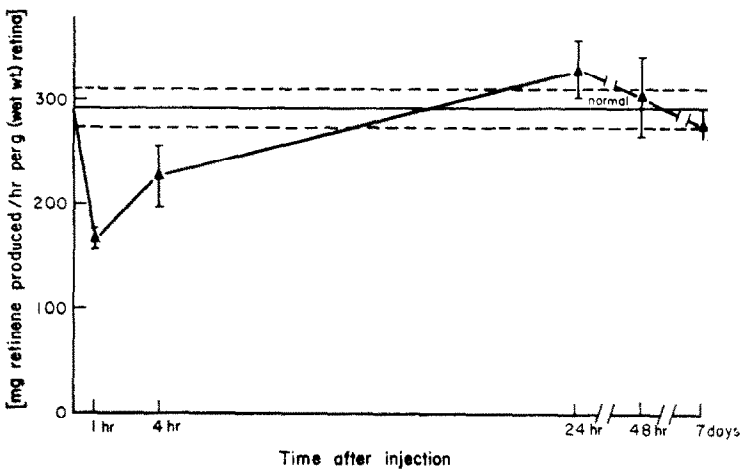


FIG 1. Effect of 1:5-di-(*p*-aminophenoxy) pentane dihydrochloride (5 mg/kg, i.v.) on retinene reductase in rabbit retina. ○, Normal value (no drug administered). ▲, After administration of drug. S.D. of each value is shown by horizontal lines.

Effects on the visual cycle in the rabbit

The effects of a single intravenous injection of 5 mg/kg 1:5-di-(*p*-aminophenoxy) pentane on the visual pigment content of the rabbit retina are recorded in Tables 2 and 3.

Table 2 refers to the effects under conditions of dark adaptation, whilst Table 3 refers to the effects under conditions of light adaptation. Visual pigment content is expressed as μg retinene/g (wet wt.) of retina, all values being the mean of three determinations.

Futterman and Saslaw[13] showed conclusively that the visual purple (rhodopsin) content of digitonin extracts of retinae is directly proportional to their retinene content, and that the former can be estimated using the thiobarbituric acid assay method for retinene.

In the dark-adapted animal (Table 2) injection of the drug produced a reduction in visual pigment content of the retina, even when the injection was carried out under the light of a red darkroom lamp and the animal was then left in the dark for 1 hr

TABLE 2. EFFECTS OF INTRAVENOUS INJECTION OF 1:5-DI-(*p*-AMINOPHENOXY)PENTANE DIHYDROCHLORIDE (5 mg/kg) ON VISUAL PIGMENT CONTENT OF RABBIT RETINA

Conditions	Visual pigment content of retina (μg/g)
Untreated animal, dark-adapted for 1 hr	25·7
Animal dark adapted, drug injected in dark, animal kept in darkroom 1 hr then estimation carried out on retina after enucleation	21·8
Animal dark-adapted, drug injected, then animal subjected to strong light for 1 hr before estimation (bleaching)	5·8
Untreated rabbit subjected to light for 1 hr (bleaching)	9·1

Results expressed as μg retinene/g (wet wt.) of retina.

TABLE 3. EFFECTS OF INTRAVENOUS INJECTION OF 1:5-DI-(*p*-AMINOPHENOXY)PENTANE DIHYDROCHLORIDE (5 mg/kg) ON VISUAL PIGMENT CONTENT OF RABBIT RETINA

Conditions	Visual pigment content of retina (μg/g)
Untreated rabbit, subjected to strong light for 1 hr (bleaching)	9·1
Rabbit under strong light for 1 hr, drug then injected, kept in light for further 1 hr. Estimation then carried out.	8·0
Similar conditions but visual pigment estimation carried out 24 hr after injection. (Rabbit kept in continuous illumination for 24 hr)	9·1
Rabbit under strong light for 1 hr (bleached), drug injected, then dark-adaptation for 1 hr	14·8
Untreated rabbit, dark-adapted for 1 hr	25·7

Results expressed as μg retinene/g (wet wt.) of retina.

before assay. This reduction amounted to about 15 per cent. Furthermore, when the drug was administered to the dark-adapted animal and this was followed by light adaptation, the visual pigment content of the retina was reduced to well below that obtained by similar light adaptation in control animals.

In the light-adapted animal (Table 3), injection of the drug did not appear to produce any further decrease in visual pigment in the retina. However, the drug did cause impairment of the ability of the retina to re-synthesize visual pigment during subsequent dark adaptation. The inhibition in this case amounted to about 42 per cent.

DISCUSSION

Inhibition of retinal alcohol dehydrogenase activity, (the enzyme responsible for the reduction of all-trans-retinene to vitamin A alcohol) concomitant with a tendency

towards a decrease in HMP shunt activity in the retina following the injection of 1:5-di-(*p*-aminophenoxy)pentane are of interest, since the two pathways are metabolically "coupled" in the normal functioning of the mammalian visual cycle. It would appear from the present results that the effects on HMP shunt activity are not as rapidly observed as those on retinal alcohol dehydrogenase, nor are they so conclusive. This is probably due to the fact that the "coupling link" is the pyridine nucleotide co-enzyme NADP/$NADPH_2$ and for a marked effect on the HMP shunt to appear, a definite and rapid shift in the intracellular redox potential to alter the $NADPH_2$/NADP ratio must also occur.

The fact that both metabolic processes are affected, and in a predictable manner, is confirmation of a specific effect of the drug on this aspect of the visual process.

The most interesting aspects of the effects of the drug on the retina were those observed on the stability of the visual pigment, especially after dark adaptation. Under such conditions, even when the drug was administered in the dark, a substantial reduction in visual pigment content was observed whilst the animal was still under dark adaptation conditions. Such an effect was not observed after bleaching (Table 3). Injection of the drug did not reduce the amount of residual visual pigment estimable even after 24 hr continuous illumination.

However, the drug did affect the capacity of the retina to re-synthesize visual pigment after bleaching.

One explanation of the above phenomena could be that some of the mammalian visual pigment exists in a non-functional state and that 1:5-di-(*p*-aminophenoxy) pentane affects intact visual pigment only in its functional state. Dowling[14] has shown that even after prolonged bleaching there is always some residual visual pigment present in the retina. This amount varies between 15 and 30 per cent of the total (dark-adapted) quantity of visual pigment. This fraction cannot be functional in the physiological sense, since it cannot initiate impulses along the optic pathway. It is significant that the quantity of visual pigment lost after drug administration in the dark (Table 2) is almost the same as the difference between the amounts estimable after bleaching with and without drug administration (Table 2). If one accepts the argument that only "bound" or functional visual pigment is affected by the drug, then this explains why no further loss in visual pigment occurs on drug administration to animals with "bleached" retinae. The protein of the "bound" fraction of the visual pigment could have conformational differences from the "residual" moiety fraction.

In summary therefore, it would appear that 1:5-di-(*p*-aminophenoxy)pentane which causes acute retino-toxicity will affect at least two biochemical systems associated with the visual cycle, viz.: retinal alcohol dehydrogenase and the stability of the visual pigment, itself. The inhibition of alcohol dehydrogenase and the concomitant reduction in HMP shunt activity will tend to inhibit re-synthesis of the visual pigment, since this metabolic cycle is associated with both reduction and oxidation of vitamin A alcohol. In fact, it might be suggested that these effects point to the target site of action of the drug on the retina as being in the outer segments of the rod cells, since retinal alcohol dehydrogenase was recently shown by Koivusalo and Tarkkanen[15] in histochemical studies to be situated in the distal parts of the visual cells of cattle and rat retinae. The visual pigment in its functional form is also situated in the lamellae of the outer segments of the rod cells.

It has also been shown that 1:5-di-(*p*-aminophenoxy)pentane produces protein

denaturation effects in the retinae of rabbits.[16] This effect is manifest by an "unmasking" of protein-SH (thiol) groups. The role of thiol groups in the retina is still subject to some speculation, but Wald and his colleagues[17] have shown that the action of light in the bleaching of visual pigment is associated with the "unmasking" of thiol groups, two per molecule of visual pigment. In addition alcohol dehydrogenase is well known to be dependent on intact thiol groups for its activity and is particularly susceptible to inhibition by thiol-group inhibitors. Therefore, a possible explanation of the marked and highly specific toxic effect of 1:5-di-(*p*-aminophenoxy)pentane on retinal function and structure lies in the fact that both retinal alcohol dehydrogenase and the visual pigment are dependent on intact thiol groups for their activity and also that these two proteins are structurally closely integrated in retina.

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