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Structural and functional alterations in the rat retina after long term exposure to two n-hexane metabolites

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List of Papers

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals

I Bäckström B, Collins VP (1987) Cytoskeletal changes in axons of rats exposed to 2,5-hexanediol, demonstrated using monoclonal antibodies. *Neurotoxicol* 8: 85-96

II Bäckström B, Dumanski JP, Collins VP (1990) The effects of 2,5-hexanedione on the retina of albino rats. *Neurotoxicol* 11: 47-55

III Bäckström B, Collins VP (1992) The effects of 2,5-hexanedione on rods and cones of the retina of albino rats. *Neurotoxicol* 13: 199-202

IV Bäckström B, P Nylén, M Hagman, A-C Johnson, V P Collins, G Höglund. Effect of exposure to 2,5-hexanediol in light and darkness on the retina of albino and pigmented rats. I. Morphology. *Arch Toxicol* 1993, 67:277-283.

V Nylén P, B Bäckström, M Hagman, A-C Johnson, V P Collins, G Höglund. Effect of exposure to 2,5-hexanediol in light and darkness on the retina of albino and pigmented rats. II. Electrophysiology. *Arch Toxicol* 1993, 67:435-441.

VI Bäckström B, Shibata E, Nylén P, Collins VP (1998) 2,5-Hexanedione concentrations and morphological changes within the eye of albino rat. *Arch Toxicol* 72:597-600.

Definitions

Antagonism	The effect of the combined exposure is less than predicted.
Interaction	Any situation in which there is evidence for one agent influencing the response to the other.
Mixed exposure	Any set of two or more agents regardless of their sources that may jointly contribute to toxicity in the target population. It is thus not necessary that the exposures to the individual agents occur simultaneously.
Non-additive effect	A difference (e.g. $p < 0.05$) between the observed effect and the predicted effect (see below) at combined exposure, i.e. synergism or antagonism.
Predicted effect	The effect of combined exposure to two agents is predicted by addition of the effects of the individual exposures, using a two way factorial model.
Potentiation	The combined effect is synergistic but only one agent is effective at individual exposure.
Synergism	The combined effect is greater than predicted (see predicted effect)

Abbreviations

A	atropine
2,5-HDol	2,5-hexanediol
2,5-HD	2,5-hexanedione
MBK	methyl -n-butyl ketone
MEK	methyl ethyl ketone
NF	neurofilament
NFP	neurofilament protein
CYO	cytochrome oxidase
EM	electron microscope
ERG	electroretinogram
GLC	ganglion cell layer
INL	inner nuclear layer
IPL	inner plexiform layer
IS	inner segment
mM	millimolar
nm	nanometer, 10^{-9} meter
nM	nanomolar
ONL	outer nuclear layer
OPL	outer plexiform layer
OS	outer segment
ppm	parts per million
RPE	retinal pigment layer
SAS	Statistical Analysing System, a computer programme for a statistic analysis of data (Cody et al., 1987)
VER	Visual evoked response

Content

1. Introduction

1.1 n-Hexane

The aliphatic hydrocarbon n-hexane (Fig. 1) is used as solvent and thinner in e.g. the shoe, glue, and petroleum industries (motor fuel contains about 1- 5 % n-hexane and industrial benzine to about 50 %), in extraction of vegetable oil, in dry cleaning, in the production of polyethylene, and polypropylene and also in many research laboratories. Extensive n-hexane exposure may occur among “glue sniffers” (Altenkirch et al., 1977; Korobkin et al., 1975). It is taken up via the lungs and excreted through the lungs and kidneys. n-Hexane is metabolised by the liver (Fig. 2). The n-hexane metabolites used in these investigations were 2,5-hexanedione (acetonylacetone; 2,5-HD) and 2,5-hexanediol (2,5-HDol). Both substances are colourless, viscous liquids that are easily soluble in water. Of the metabolites, 2,5-hexanedione (2,5-HD) is believed to be the most neurotoxic (Spencer et al., 1980).

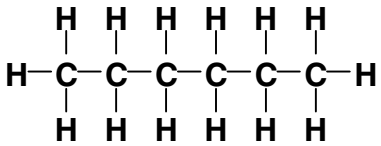

Figure 1. Chemical structure of n-hexane

1.2 Neurotoxicity

Acute n-hexane toxicity includes narcotic effects such as dizziness, headache, euphoria, intoxication etc. Chronic effects were observed in 1976, when Schaumburg and Spencer reported that pure n-hexane exposures produce clinical and pathological evidence of peripheral neuropathy (Schaumburg et al., 1976). Commercial hexane mixtures include many different substances. A n-hexane mixture free from the n-hexane did not give rise to neurotoxic effects (Egan et al., 1980). A number of following studies indicated that n-hexane is the chemical responsible for the polyneuropathy occurring in humans repeatedly exposed to high concentrations of commercial hexane solvents (Cavanagh, 1982a; Cavanagh, 1982b; Cavanagh et al., 1981; Jones et al., 1982a; Jones et al., 1982b; Schaumburg & Spencer, 1976; Spencer et al., 1980). Methyl n-butylketone, which is also partly metabolised to 2,5-HD has been reported to produce similar neurological syndromes in man and experimental animals (Spencer et al., 1980).

Methyl ethyl ketone (MEK) is a common contaminant in commercial hexane products (Altenkirch et al., 1979). MEK alone does not produce signs of neurotoxicity but potentiates the neurotoxicity of n-hexane in rats (Altenkirch et al., 1978; Altenkirch et al., 1982). Acetone, frequently present in hexane products enhances the neurotoxic effect of the n-hexane metabolite 2,5-HD (Ladefoged et al., 1984). Toluene reduces the neurotoxicity of n-hexane in rats probably by reducing the formation of toxic metabolites of n-hexane (Nylén et al., 1989; Takeuchi et al., 1981).

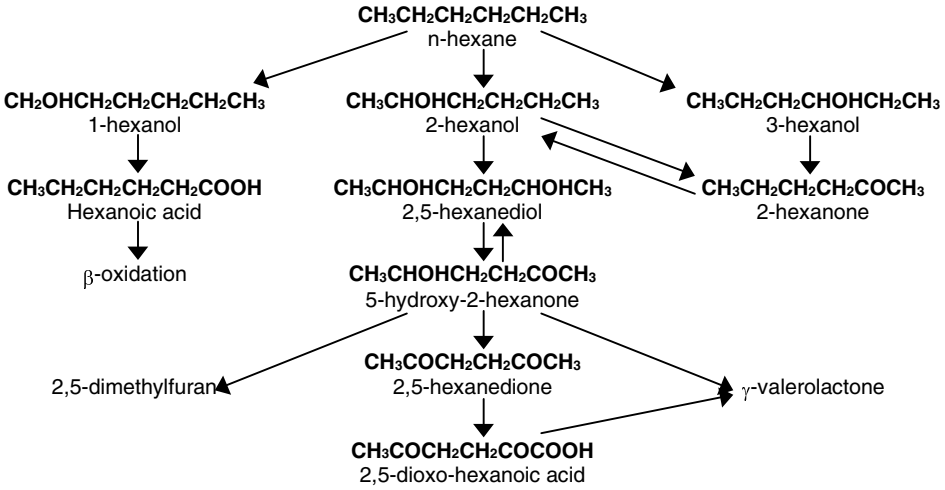

Figure 2. Metabolic pathway of n-hexane, modified from Ladefoged, Arbete och Hälsa 1986:20.

n-Hexane is first metabolised in the liver to 2-hexanol through the microsomatic monoxygenase system (cytochrome p-450) and thereafter by alcoholdehydrogenase to the metabolite 2,5-hexanediole (2,5-HDol) and further to 2,5-hexanedione (2,5-HD), both used in the present studies. The most neurotoxic of these metabolites is 2,5-HD (Spencer et al., 1980). 2,5-HD produces swellings proximal to the nodes of Ranvier at the ends of long nerve axons (Cavanagh & Bennetts, 1981). Such swellings contain neurofilament proteins (NFP) which are of three kinds determined by the molecular weight: 68, 160 and 200 K Dalton. The toxic changes are characterised morphologically as a central-peripheral distal axonopathy with distal and retrograde damage occurring specifically in long and large fibertracts of the central and peripheral nervous systems. The sciatic nerve is affected early in humans and may ultimately cause paralysis. The damage of the distal axon proceeds toward the nerve cell body, initially resulting in clinical signs in the feet and hands, called stocking-glove sensory loss. Affected regions in the brain are the optic pathways, lateral lemniscus, some parts of the cerebral cortex,

cerebellar cortex, and cerebellar tracts. The swellings of the axons are reversible when exposure to n-hexane or n-hexane metabolites is ceased. Axonal regeneration is slow and recovery may take months or years, with some paresis frequently remaining after heavy exposure.

Different theories exist about how the axonal swellings arise. Some studies suggest that the swellings may start when the metabolite of n-hexane, 2,5-HD, hastens the axonal transport resulting in an accumulation of neurofilaments (NF) at the nodes of Ranvier in the distal part of long nerves (Monaco et al., 1990). The neurofilaments in the axons are then suggested to react with 2,5-HD to form pyrrol substances (Graham et al., 1980). Such products have also been identified in the urine and serum (Ogata et al., 1991). A recent study casts a doubt on the role of swelling as primary cause of degeneration (Sickles et al., 1994). It was instead suggested that the neuronal alterations are formed by pyrrol substances (Graham, 1997). Another theory suggests that the serious weight loss that occurs when animals are exposed to 2,5-HD may produce the axonopathy due to a reduction of energy production (Medrano et al., 1989). The authors also showed that the hexane metabolites in all brain regions produce a substrate-dependent inhibition of brain mitochondrial respiration. This inhibition of energy production might play a role in the neurotoxic mechanisms of action of these chemicals. Many authors (Cavanagh, 1982a; Cavanagh, 1982b; Cavanagh & Bennetts, 1981; Jones & Cavanagh, 1982a; Jones & Cavanagh, 1982b; Schaumburg & Spencer, 1976; Spencer et al., 1980) have described how both man and laboratory animals show pronounced weight loss after exposure to n-hexane or its metabolites. It has not been determined if this weight loss, the seriousness of which depends on the total dose of the hexacarbons, may influence the paralysis.

1.3 Other organs showing toxic reactions

Exposure to high concentrations of n-hexane causes testicular atrophy in albino rats. Testes are badly damaged during exposure to n-hexane and its metabolites with degeneration of the seminiferous tubules and loss of germ cells. After a recovery period testicular function may be resumed (Boekelheide, 1987; Boekelheide et al., 1989; Cavanagh, 1982a; Nylén et al., 1989).

After exposure to n-hexane, blurred vision has been reported. No evidence of visual loss has, however, been documented (Korobkin et al., 1975; Spencer et al., 1980). Raitta et al. have reported abnormalities in the electroretinogram in man that may reflect macular dysfunction(Raitta et al., 1981). The same study showed that workers exposed to n-hexane had difficulties in perceiving the blue-yellow axis of the spectrum. It has been claimed (Chang, 1990) that 2 of 11 patients with n-hexane induced polyneuropathy also had abnormal colour vision and maculopathy. No regeneration was documented after several years of recovery without exposure to n-hexane. Reversible damage to the optic tract resulting in temporary visual loss has been observed in macaques treated with 2,5-HD

(Lynch et al., 1989). No pathological examination of the photoreceptor cells was performed.

The blood-aqueous humor barrier is an important restraint for free solutes in the blood to enter the chambers of the eye. Permeable walls of the vessels of the ciliary body are blocked by tight junctions that connect special cells in the ciliary body. These tight junctions are believed to represent the major anatomical site of the blood-aqueous humor barrier. The aqueous humor resembles the cerebrospinal fluid. It contains e.g. ascorbate in a concentration about 25 times higher than in blood. There is a continuous secretion of solutes by the ciliary epithelium probably through a process of active transport.

No evidence of mutagenic, carcinogenic, or teratogenic effects of n-hexane and its metabolites (2,5-heaxanedione and 2,5-hexanediol) has been reported. Interestingly, no data of photoreceptor cell damage after exposure to n-hexane or its metabolites had been found previously to the studies presented in the present thesis.

2. Some aspects of eye anatomy, function and photoreceptor cells

The eye is very schematically described in Fig. 3. The retina consists of photoreceptor cells, neurones of different sorts, synapses, supporting structures, and a unicellular pigmented layer (Fig. 4). Half of the depth of the retina consists of the photoreceptor cells, the rods and cones (Fig. 5).

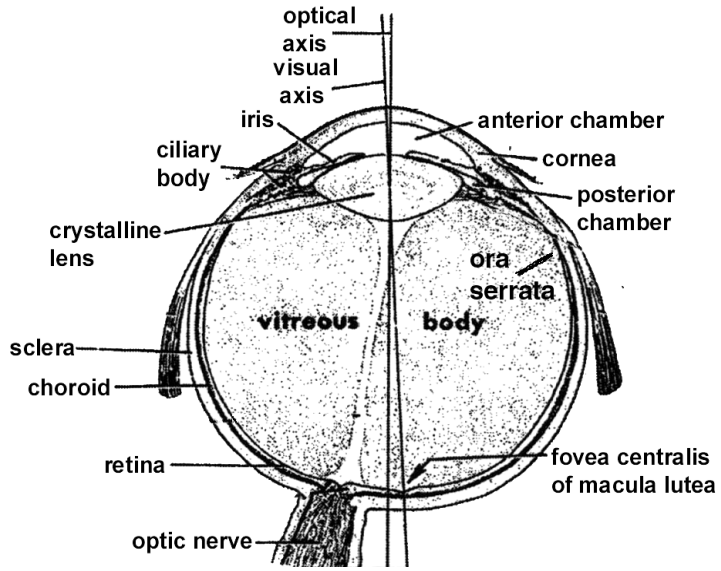

Figure 3. Schematic section through the right eye (horizontal plane). Modified from Crouch, Functinal human anatomy third ed., Lea&Febiger, Philadelphia 1978.

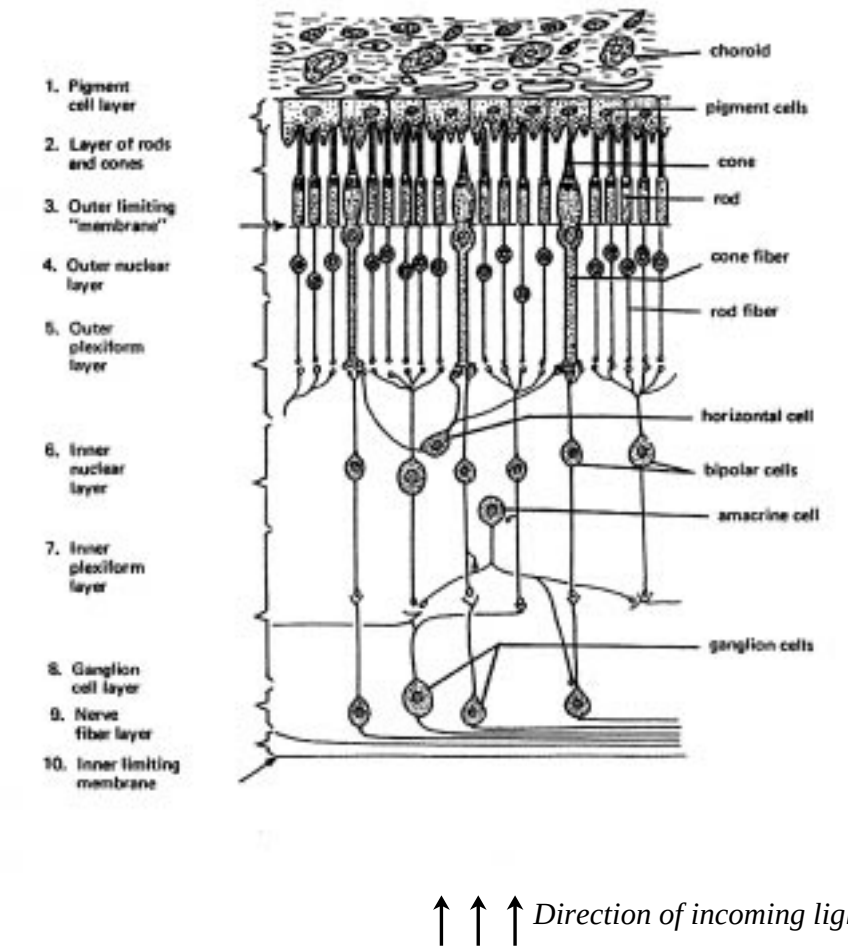

Figure 4. Cellular components of the retina. Modified from Crouch, Functinal human anatomy third ed., Lea&Febiger, Philadelphia 1978.

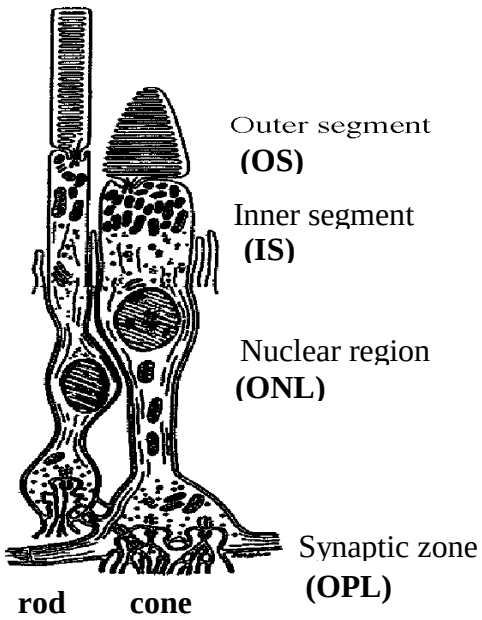

Figure 5. Photoreceptor cells. Modified from Missotten: The ultra structure of the human retina. Brussels; Belgium. Copyright by editions ARSCIA, 1965.

In humans, the cones are concentrated in a small area, macula lutea, near the penetration of the optic nerve. The rods and cones in rats are, however, spread over the whole retina. Nerve impulses are initiated in the photoreceptors and transformed through the outer plexiform layer (OPL) to the bipolar cells. The axons of bipolar cells synapse with the dendrites of ganglion cells, whose axons form the optic nerve, at the posterior end of the eye ball (Fig. 4). There are some other nerve cells, e. g. the amacrine and horizontal cells, that together with the bipolar cells constitute the inner nuclear layer (INL) and inner plexiform layer (IPL). The retina ends near the cornea in a jagged margin called ora serrata.

The cones are of three kinds, each absorbing light from the blue, green or red regions of the spectrum (Fig. 6). Their optimal function is within bright light. The rods, that represent the black-white scale of vision, contain a highly light-sensitive chromophore, rhodopsin. Rhodopsin is made up of the protein opsin, bound through the aminoacid lysine, to the chromophore retinal.

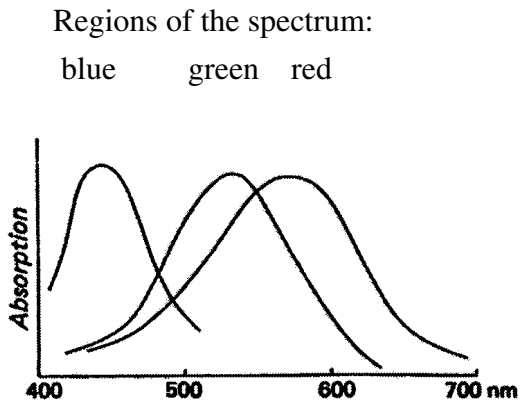

Figure 6. Relative absorption spectrum of cones

Rhodopsin absorbs photons from all visible wavelengths with the maximum of absorption at about 523 nm. Rats are nocturnal animals, which explains the high proportion of rods (97%).

3. Light induced damage to the retina – suggested mechanisms

3.1 Blue light induced damage

The conditions that usually produce damage to the retina are photochemical rather than thermal. Special substances called chromophores absorb photons of different wavelengths in the visible light region. Each chromophore has its own absorption characteristics by virtue of its molecular structure and can therefore only absorb photons of specific energies, e. g. radiation of specific wavelengths. The shorter the wavelength is, the greater the potency of damaging tissue. Violet and blue lights, thus, cause most injury. In experimental studies it has been shown that blue light may damage the retina of rats in a dose-dependent manner (Chen, 1993). At about 440 nm blue cone damage is induced through absorption by iodopsin, a violet pigment, in some retinal cones, and by cytochrome oxidase in the mitochondria of photoreceptor cells. Melanin in the retinal pigment epithelium may catch photons in the blue spectral area and act as a protective substance. Absorption of optical radiation by chromophores can also induce photochemical reactions (e.g. mitigating the visual process).

3.2 Phototoxic reactions

When photons are absorbed by chromophores two states of energy may arise, the higher Singlet State or lower Triplet State, depending on if the electron spin changed. A photochemical reaction has occurred, when the pathway for energy dissipation does not include return to the starting molecule configuration. Many types of photochemical reactions involve alteration in one molecule as a result of absorption of photons by other molecules. These latter reactions are called photosensitised reactions and the absorbing chromophore is then referred to as the photosensitizer.

3.3 Type I and II reactions

The photons absorbed by photosensitizers will excite these to the Singlet State (Fig 7). The life span of this state is very short and will through intersystem crossing usually go into the lower energy state, the triplet, that lasts longer. Photoreactions starting from the Triplet State can be classified in two groups, Type I and Type II reactions.

In Type I reactions, there is a direct reaction between the triplet photosensitizer and molecules in its vicinity by a transfer process of an electron or hydrogen atom. The sensitizer of Type I may abstract an electron from a reducing substrate

to produce chemically reactive free radical products. These free radicals can react with oxygen in its ground state (the Triplet State) to form superoxide, which is most likely to damage the plasma membrane. Nucleic acid damage and protein cross-linking may result from Type I reactions.

Type II reactions are indirect reactions and the triplet photosensitizer commonly reacts with molecular oxygen producing a highly reactive singlet oxygen. This may interact with polyunsaturated fatty acids to form e.g. lipid peroxides.

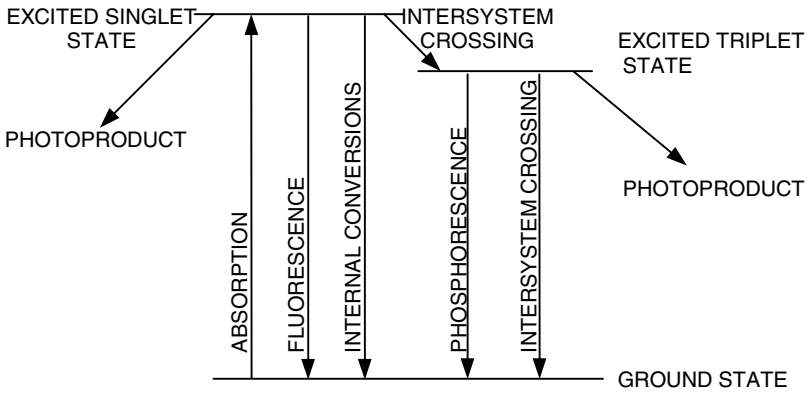

Figure 7. Reactions of photons. Modified from Dayhaw-Barker et al.:Optical radiation and health, CRC press Inc. Boca Raton Florida 1986.

3.4 Free radicals

The free radical species from Type I reactions may accordingly be formed in light induced reactions when light of short wavelengths, i.e. violet-blue light, is absorbed by the retina and may rapidly lead to the destruction of cell components. This damage also includes alteration of structural components, genetic material and membranes through lipid peroxidation and cross-linking reactions. The presence of antioxidants such as vitamin C or E, helps to quench free radical species. If the generation of free radicals exceeds the detoxification system, cell damage or cell death may be the consequence. It is known that melanin offers protection to light induced damage to the eye tissue because of its ability to catch radicals, formed in light. The radicals can also be formed in darkness (no radiation) reactions.

3.5 Enzyme interactions

Hansson observed a decrease e.g. in the activity of cytochrome oxidases, a family of mitochondrial enzymes, in the rat retina after exposure to light from fluorescent

tubes (Hansson, 1970). Most cytochrome oxidases are situated in the inner segment layer and epithelium cells of the retina. The inactivation of these respiratory enzymes was followed by cellular damage. As mentioned before, cytochrome oxidase absorbs photons in the blue region of visible light that is most energy rich and is able in vivo to inhibit cytochrome oxidase and thus damage retinal cells (Chen, 1993). It is further known that cytochrome oxidase activity is decreased after exposure to 2,5-HD (Spencer et al., 1980). Cytochrome oxidase might therefore be a site of interaction between light and 2,5-HD.

4. Aims of the study

1. To investigate whether immunohistochemical techniques with monoclonal antibodies against neurofilament proteins can be used also to evaluate the axonal abnormalities induced by 2,5-HD or 2,5-HDol.

2. To document whether there is a surplus of tubulin in addition to neurofilament in the axonal swellings of 2,5-HD or 2,5-HDol treated rats by using an immuno-histochemical method.

3. To screen some major organs of 2,5-HD or 2,5-HDol exposed rats for neuronal damage.

4. To investigate in detail the morphology of the retina and the optic tracts following 2,5-HD or 2,5-HDol exposure.

5. To study the influence of simultaneous exposure to light on 2,5-HD or 2,5-HDol induced alterations on the rats retina.

6. To study if any light induced increase in such retinal damage (aim 5) progresses after the end of exposure to 2,5-HD or 2,5-HDol.

7. To analyse if 2,5-HD penetrates the blood/retinal, aqueous humor barriers.

5. Material and methods

5.1 Animals

Male albino rats, (Sprague-Dawley), were used in all investigations. In studies IV and V, also pigmented rats, Norwegian Brown, were used. The animals weighed 200-220 g (albino) or 100-150 g (pigmented) at the start of the experiment. The pigmentation of the iris in pigmented rats would be expected to attenuate the amount of light reaching the retina (Rapp et al., 1980) and hence to reduce the risk of light induced damage of the visual cells. These different reactions to n-hexane metabolites made it necessary to expose the pigmented rats about 3 weeks longer.

Since the suspicion arouse that the loss of photoreceptor cells partly depended on exposure to light energy, one main group of rats was exposed to 2,5-hexanedione or 2,5-hexanediol in light and the other group was housed in total darkness. This protocol was used to study if light was necessary produce the damage of the photoreceptor cells. The dark room had a lock-chamber and red dim light lamps were only on when taking care of the animals. After about 5-6 weeks half of the exposed and the control rats were sacrificed. The exposure of the remaining rats was allowed to continue for 5 weeks or more under the same conditions but without exposure to 2,5-HD or 2,5-HDol.

Albino and pigmented rats were divided into 4 groups in the later studies (papers IV, V). One group was exposed to 1 % 2,5-HDol only, one to light only, one to both 2,5-HDol and light, and the remaining group was kept in darkness without exposure to 2,5-HDol. After 5 weeks (albino rats) and 8 weeks (pigmented rats) half of the rats were sacrificed and the rest were allowed to recover during a 13 week period without exposure to 2,5-HDol. The studies were approved by North Stockholm Animal Ethics Committee of Sweden.

5.2 Exposure to 2,5-hexanediol

Rats exposed to 2,5-hexanediol or 2,5-hexanedione received the substance as a tap water solution (ad libitum). These solutions were the only liquids available to the animals until the administration of the solvents was terminated after 5 or 6 weeks for the albino rats. The reason for ending the exposure was severe hind limb paralysis in most albino animals and after 8 weeks for the pigmented rats when most of the animals had a paddling gait as the major neurological sign. The solutions were freshly prepared and changed 3 times weekly. Since the intake of n-hexane metabolites together with water was very low the rats were administered 2,5-HD through tube feeding in a later Paper (VI) in order to avoid the bad taste of the n-hexane metabolites. The control groups were tube fed by tap water only.

5.3 Light exposure

The rats in Paper I-III were caged individually, placed on racks, circulated according to a rotation protocol, thus receiving the same quantity of light energy from 4 standard fluorescent tubes (Thorn EMI T40W/29). The cages were exposed to a luminance of 200, 55, 45, 35, 25 and 15 cd/m^2. The light exposed rats in Paper IV-VI and the referred atropin study were placed 4 to6 rats in each cage on shelves 2.0 m below the same fluorescent tubes and were exposed to a light/dark cycle of 12/12 h. In later studies the luminance inside the cage varied with cage location between 25 and 35 cd/m^2. The total long term light exposure was, thus, close to identical in all cages. The light conditions prevailing for each group were maintained during the period when no group was exposed to n-hexane metabolites. To find out if 2,5-HD or 2,5-HDol had a toxic effect of its own the rats were housed in darkness and exposed to dim red light only during the daily care.

5.4 Observation protocol

Weight gain, water consumption, appearance, and neurological status as observed behaviourally, were recorded weekly in all studies, and stages of clinical signs were documented.

5.5 Histology

Half of the animals in each group was sacrificed immediately after the completion of exposure to 2,5-HD or 2,5-HDol. The animals received a lethal dose of thiopental sodium, immediately followed by perfusion fixation using B5 (solution A: 12 g $HgCl_2$, 2.5 g NaAc, 200 g H_2O; solution B: 40 % Formaldehyde; working solution: B5 = 20 ml sol. A + 2 ml sol. B;). The excised materials (brain, nerves, eyes, other tissues) were kept in B5 for 40 minutes, and then stored in ethanol (70%) until embedding in paraffin (the rats in studies V and VI were not perfusion fixed). This technique was used when performing brain and nerve preparations. The remaining rats were sacrificed after the recovery periods and the same material were treated in an equivalent manner.

The right eye was divided into two equal halves by a horizontal section through the optic nerve and cornea. The lens and the vitreous body were removed. The rest of the eye was embedded in paraffin, sectioned (thickness 4 µm) perpendicularly to the retinal surface, stained with hematoxylin-eosin and examined by light microscopy.

5.6 Morphological measurements

The number of nuclei in the ONL (the nuclei of photoreceptor cells) was assessed using a "MOP" Videoplan equipment (Kontron image analysis systems; Carl

Zeiss, Germany) by counting the nuclei in segments of retina, 100 μm in length. Four or five sequential adjacent segments progressing peripherally in a medial as well as a lateral direction from a point 200 μm on each side of the optic nerve were studied, i.e. 8 to 10 segments were observed in each histological section. In addition, the sections were examined for morphological changes. This arrangement has been recommended (Fox et al., 1988) since measuring of the different layers in the retina may give uncertain values because of difficulties in preparation of the eyes.

The relative thickness of the outer plexiform layer (OPL) was calculated as the thickness (μm) of the OPL in the segment divided by the thickness (μm) of the (OPL) + inner nuclear layer (INL) + the inner plexiform layer (IPL) + the ganglion cell layer (GCL), thirteen weeks after the end of the exposure to 2,5-HDol. This method was used in order to avoid overestimation of the depth of the OPL due to possible non-perpendicular cutting of the retina.

5.7 Electrophysiological measurements

The electrophysiological measurements comprised electroretinography (ERG), visual evoked response (VER) and pupil recordings, methods that are described in detail in paper V.

ERG. The rat was kept in darkness 12 h before registration. The pupil was dilated and the light stimulus was generated by a xenon lamp and guided by fibre optics to the eye. Recordings were made using a corneal contact lens. The corneal lens and vertex of the skull were connected to a pair of calomel electrodes by plastic tubes filled with saline. An average of five recordings was used for calculation of latency and amplitude of a-, b-, and c-wave.

VER. Subcutaneous needle electrodes were placed in the midline above the visual cortex and 5 mm rostral to the bregma. The flash stimulus was generated by a photostimulator. The peak-to-peak amplitude and the latencies of two prominent peaks were calculated from an average of 64 recordings.

Pupil diameter. Light reached the cornea of an dilated eye at an angle of 45 degrees from the vertical axis. The diameter of the pupil was determined by a vernier calliper and a dissection microscope. This measurement was made immediately after the electrophysiological recordings.

5.8 Analysis of 2,5-HD and analytical procedure

The analysis and analytical procedure of 2,5-HD are described in detail in Paper VI. To reduce the high pressure in the eye chambers the eyes were frozen to -80°C. Most of the procedure was based on the methods originally developed by Kezic and Monster (Kezic et al., 1991) and modified by van Engelen et al. (van Engelen et al., 1995). To each sample 200 ml of citrate buffer (pH 2.2), 50 ml of

2,4-pentanedione (16.23 mM) as an internal standard, and 50 ml of O-(2,3,4,5,6-pentafluorobenzyl)-hydroxylamine hydrochloride solution (80 nM) was added together with 1.5 ml of cyclohexane. The cyclohexane phase was injected into a Varian model 3700 Gas Chromatograph with electron capture detector. The electron capture detector facilitated the detection of the small quantities of 2,5-HD found in the eyes after the exposures.

5.9 Immunohistochemical techniques

Electron microscopy and conventional silver impregnation, the usual techniques to studying the changes in axons, have their draw-backs; and do not identify the molecular components of a pathological change following exposure to n-hexane and its metabolites. Thus, a new technique (Collins et al., 1983) using monoclonal antibodies against neurofilament or tubulin was tried. The material was incubated with mouse monoclonal antibodies against neurofilament protein or tubulin. Peroxidase conjugated rabbit antimouse Ig antibodies were used as a second antibody. The antibody complex was then coloured brown by diaminobensidin (DAB). The techniques is described in Paper I.

5.10 Statistical methods.

Statistical comparisons between groups, using Statistical Analysis System, SAS (Cody and Smith 1987), were made by one-way analysis of variance (ANOVA) followed by Duncan´s multiple range test. Behavioural sign scores were studied using Wilcoxon rank sum test. Interactions were tested using a two-way factorial model (GLM; General Linear Models). Observations on groups were considered as "different" only if the probability for no difference was <0.05, unless otherwise mentioned. In order to exclude an effect of possible age related changes in retinal morphology, statistical comparisons were made only between groups of the same age.

6. Results

In Paper I, albino rats were exposed to 2,5-HDol for 6 weeks, followed by two post-exposure periods of 5 or 10 weeks. At the end of exposure, the rats showed severe neurotoxicological symptoms e. g. paralysis in the hind legs. Within the central nervous system, considerable swellings were observed within the brain tissues such as the parietal and occipital lobes, superior colliculi, lateral lemniscus, as well as the cerebellar cortex, as studied by immunohistochemical technique. Swellings (torpedoes) in the first segment of the Purkinje cells were found in the cerebellar tissue. Tractus opticus was badly damaged. No swellings were seen in the optic nerve. Parts of the peripheral nervous system, e. g. sciatic nerves, contained swollen axons in the distal parts of the nerves. In albino rats exposed to 2,5-HDol, nerves contained swollen axons in e.g. the stomach, intestines, liver, spleen, pancreas, kidneys, adrenals, lungs, heart, eyes, and salivary glands. Testes, skin, and adrenals were severely damaged. The paralysis decreased during the post-exposure periods without 2,5-HDol in 5 or 10 weeks. Nerves from the same regions of the organs were then examined morphologically. The swellings had decreased and the proportion of swollen axons was lowered.

Macroscopical examination of the 2,5-HDol treated rats revealed that the testes were smaller than normal. At sectioning a clear fluid was visible, instead of the normally found milky solution filled with sperm. Microscopically the testes showed atrophy after the end of exposure to 2,5-HDol. After a 5 weeks period without exposure to 2,5-HDol, some of the rats had recovered and after an additional 5 weeks the testicles of all rats observed in the microscope seemed to be normal.

The skin was severely damaged when examined from outside. It had brown flakes probably formed from the skin epithelium. The skin reacted immediately on drops from 2,5-HD with yellow spots. Old exposed albino rats had obtained a dirty yellow colour.

The present study showed clear damage to the retina manifested as a loss of photoreceptor cells. This loss progressed during the post-exposure periods. In order to study the eye injuries more in detail albino rats were exposed for 5 weeks to 2,5-HD, after which some of the rats were sacrificed and tissue was processed for microscopical study. The remaining rats were left for 13 weeks without 2,5-HD exposure. After the exposure to the 2,5-HD, the outer segment (OS) of the photoreceptor cells was the first layer to be affected. The segments was fringed, thinned, shorted and filled with vacuoles immediately after exposure to 2,5-HD. The inner segments (IS) were badly damaged and reduced in the most affected rats and during the post-exposure period. The nuclei (ONL) of the photoreceptor cells were pyknotic and shrunken. The 2,5-HD exposed rats showed a clear reduction of nuclei immediately after the exposure. This reduction was enhanced during the

13-week period without 2,5-HD. At the end of the period the rats had lost nearly all photoreceptor cells, except for a residue left at the ora serrata.

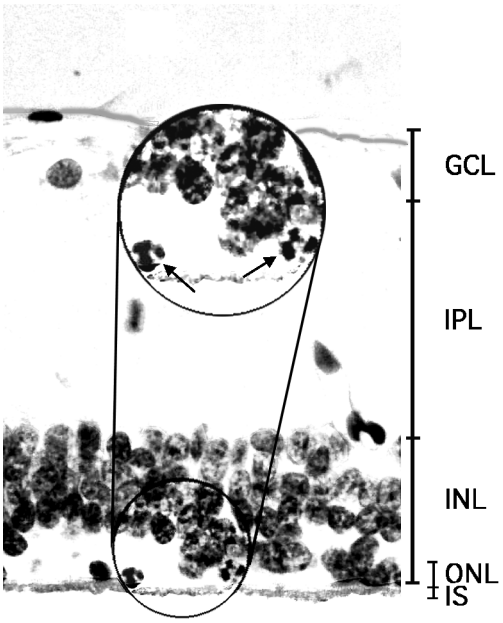

Figure 8. Retina of albino rat exposed to 2,5-HD. Note cones marked with arrows. From Paper II.

Eyes from the albino rats in Paper III were investigated concerning differences in damage and number of rods and cones. The nuclei of rods and cones were counted separately and compared. The investigation showed that the rods were significantly reduced immediately after the exposure to 2,5-HD. In contrast, there was no reduction of the cone population. After 13 weeks without 2,5-HD all the rods had disappeared but about 50% of the cones remained.

Ora serrata is the area of the retina that is most distant from the visual axis and, thus, the least light exposed. The observation that some photoreceptor cells were retained in this area suggested that the toxic effect of 2,5-HD might interact with that of light energy. In order to detect a possible interaction between the effects of light energy, originating from fluorescent tubes normally occurring in animals departments, albino and pigmented rats were exposed to 2,5-HDol in 12 h light/12 h darkness, or to total darkness. Two groups were unexposed to 2,5-HDol, one in 12 h light/12 h darkness, and the other in total darkness. The light intensity from the fluorescent tubes was reduced to half compared to Paper I in order to induce only minor retinal alterations in animals exposed to light only. This study design was intended give information whether 2,5-HDol itself had an effect on the retina. Half of the rats were sacrificed at the end of the 2,5-HDol exposure, the others

after an ensuing 13-week period without 2,5-HDol but under the same light conditions. The outer nuclear layer (ONL) that consists of the nuclei of photoreceptor cells (Fig. 8), was reduced by about 30 % immediately after a 5 week exposure to 2,5-HDol and light. No significant retinal damage was observed in the group exposed to 2,5-HDol in darkness or to light alone. Physiological investigation using electroretinography (ERG) agreed with these results except for a tendency in 2,5-HDol treated albino rats immediately after exposure in darkness toward a slight functional alteration which disappeared during the post-exposure period.

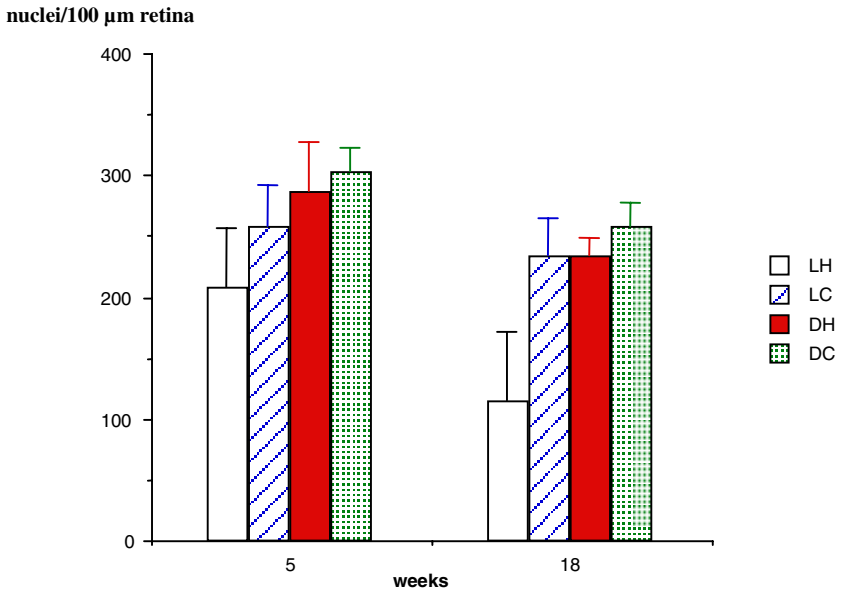

Figure 9. Retinal thickness in albino rats exposed to (LH): light and 2,5-HDol; (LC) light only; (DH) 2,5-HDol in darkness; (DC) controls in darkness; immediately after a 5 week exposure and after a 13 week post exposure period (18 weeks in total). The rats of the groups exposed to light and 2,5 HDol (LH) have a significant loss of photoreceptor nuclei after 5 weeks and a further loss after 18 weeks.

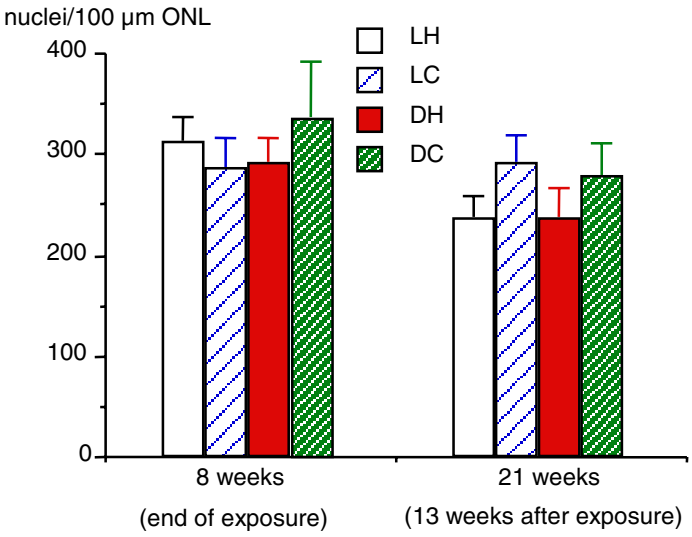

Figure 10: Nuclei/100 µm ONL in pigmented rats exposed to (LH): light and 2,5-HDol; (LC) light only; (DH) 2,5-HDol in darkness; (DC) controls in darkness; immediately after a 8 week exposure period and after a 13 week post exposure period (21 weeks in total). Rats exposed to LH or DH had a significant loss of photoreceptor nuclei after the 13-week postexposure period.

After a further 13-week period without exposure to 2,5-HDol the toxic effect on the photoreceptor cells of albino rats was more pronounced as evidenced by a loss of 50 % of ONL in the rats exposed to 2,5-HDol in light (Fig. 9). The photo-receptor cells of the albino rats, thus, were clearly damaged by exposure to 2,5-HDol in the presence of light.

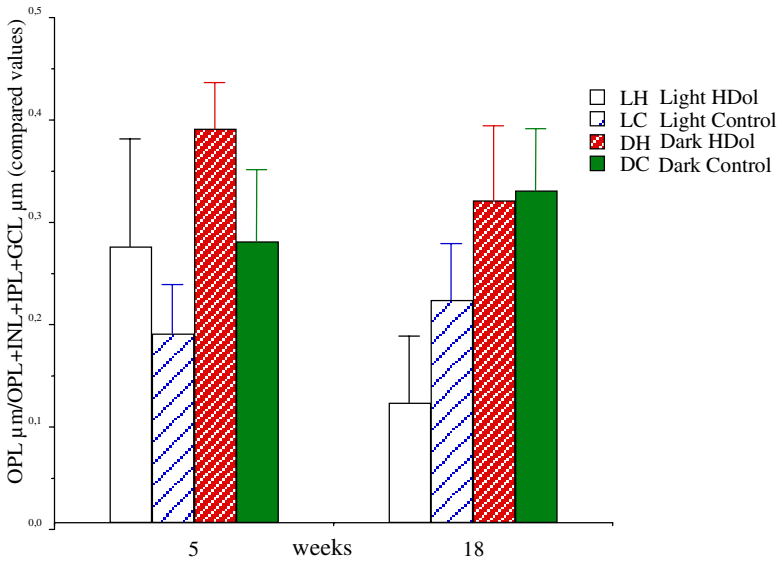

Figure 11. Relative retinal thickness in albino rats exposed to (LH): light and 2,5-HDol; (LC) light only; (DH) 2,5-HDol in darkness; (DC) controls in darkness; immediately after a 5 week exposure and after a 13 week post exposure period (18 weeks in total).The measures of the albino rats exposed to 2,5-HDol and light varied less after 18 weeks. Also the control rats in light had some loss of OPL.

The outer plexiform layer, OPL, is formed by the synapses between the photo-receptor fibres and the bipolar cell dendrites. The relative thickness of the OPL (OPL/(OPL+INL+IPL+GCL)) immediately after the exposure to 2,5-HDol varied considerably within individual sections from retinas of all albino groups, so no reliable statistical calculations could be made. Thirteen weeks later without exposure to 2,5-HDol, the OPL had less variable measures. The OPL of the rats exposed to 2,5-HDol in light was very thin, and its relative thickness was considerably less than that of the other groups. The relative thickness of the OPL was also less in the light only group than in groups exposed to the 2,5-HDol alone or kept in darkness. Reductions of the ONL and OPL exceeding a simple summation of the data recorded after exposure to light only and to 2,5-HDol only, were observed in the group exposed to2,5-HDol plus light 13 weeks after the end of the exposure to 2,5-HDol.

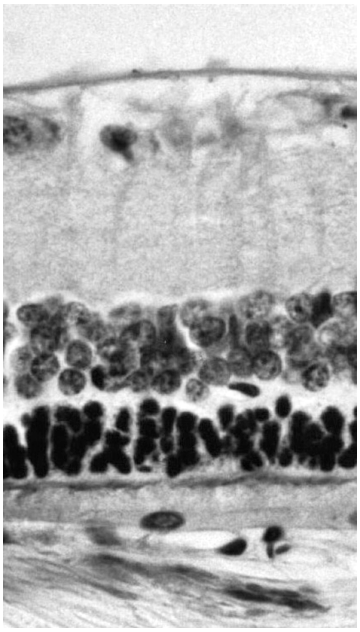

Figure 12. Sectioned retina from albino rat exposed to 2,5-HD for 5 weeks. The OS is free from structure and seem pasted to the pigment cell layer.

The number of nuclei in the ONL of pigmented rats exposed to2,5-HDol in light was not significantly different from the number of nuclei of exposed rats kept in darkness; or from unexposed rats kept in light or darkness immediately after exposure (Fig. 10). Thirteen weeks later without 2,5-HDol (Fig. 10) the pigmented rats exposed to 2,5-HDol in light or in darkness had a significant loss of photo-receptor nuclei.

The albino and pigmented rats in Paper IV were examined also in Paper V by recording the electroretinogram and visual evoked response (VER). A change in all ERG amplitudes exceeding a simple summation was recorded in the rats exposed to 2,5-HDol in light. No differences in latency or amplitude between any of the pigmented rats were demonstrated 2-5 days or 13 weeks after exposure to 2,5-HDol.

The a-wave of the ERG is generated by the photoreceptor cells after illumination of the retina. The latency 2-5 days after the end of exposure to 2,5-HDol and the amplitude 13 weeks after such exposure to 2,5-HDol resulted in a reduction exceeding a simple summation of the reductions recorded from rats exposed to 2,5-HDol in darkness and to light only.

Prolonged latency and lower amplitude were seen in the b-wave, which is believed to represent the response of the nerves of the bipolar cells in the outer

plexiform layer (OPL). The b-wave of the albino rats after 5 weeks exposure to 2,5-HDol in light showed alterations that exceeded a simple summation of the changes in rats exposed to 2,5-HDol in darkness and unexposed rats in darkness. This result is in accordance with the morphological results.

The c-wave is believed to be generated by cells forming the pigment epithelium. Changes in c-wave latency and amplitude in animals exposed to 2,5-HDol in light exceeded simple summation of alterations in rats exposed to 2,5-HDol in darkness and unexposed rats in darkness. The control rats in light showed a mild reduction of the OPL.

In order to investigate the influence of pupil widening on the loss of photoreceptor cells albino rats were treated with atropine and/or 2,5-HDol. All rats were exposed to identical light from fluorescent tubes and divided in 4 groups, one exposed to atropine; one to 2,5-HDol and one group to both atropine and 2,5-HDol. The fourth group was unexposed to both 2,5-HDol and A.

All rats in the groups exposed to 2,5-HD; to atropine, and to 2,5-HD plus atropine had a significant loss of photoreceptor cells immediately after the end of exposure. After a post-exposure period of 13 weeks the albino rats exposed to both atropine and 2,5-HDol had a significant loss of rods and cones compared to the rats exposed to atropine alone or to 2,5-HDol alone, and a further loss compared to the unexposed controls (Figure 13).

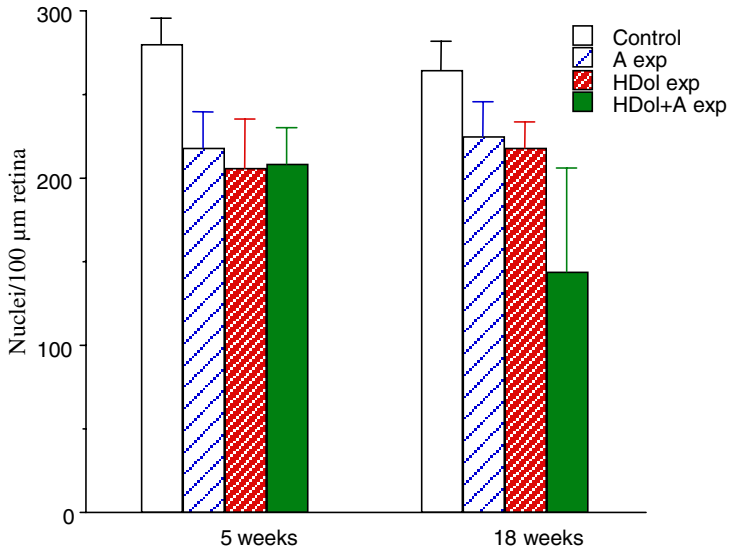

Figure 13. Number of nuclei/100 µm ONL after exposure to atropine (A), to 2,5-HDol, or to 2,5-HDol plus A, immediately after a 5 week exposure and after a 13 week post-exposure period (18 weeks in total). A significant loss of photoreceptors compared to controls was seen after exposure. Thirteen weeks after the end of exposure, only the HDol plus A group had further significant loss of photoreceptors.

Two days after the end of exposure, the pupils exposed to A were somewhat more dilated than the pupils unexposed to A. The pupils of HD exposed rats were dilated compared to those of the unexposed rats. The pupils of HD + A exposed rat eyes were most dilated (Table 1).

Table 1. Pupil diameter in mm (mean and SD)

Exposure	5 weeks	9 weeks
Control	0.93 ± 0.05	1.00 ± 0.08
Atropine	1.00 ± 0.14	1.00 ± 0.09
2,5-HD	1.24 ± 0.05	0.60 ± 0.08
A plus 2,5-HD	1.40 ± 0.09	0.60 ± 0.07

The pupil diameter was measured two days (unpublished data) after exposure to 1 drop of 1% A, to 1 ml 7% 2,5-HD/day for 5 weeks by tube feeding, and to 2,5-HD plus A. Rats exposed to A had a more open pupil compared to controls. Exposure to 2,5-HD Also dilated the pupil, In combination with A the dilatation was more pronounced. Nine weeks later, unexposed and A administered rats had about the same pupil diameter. The group exposed to 2,5-HD alone and to 2,5-HD

plus A had a similar size but their pupil diameter was smaller than that of unexposed controls and those exposed to A only.

Four weeks after exposure to HD and/or A (light conditions remaining unchanged) the pupil of the A group had the same diameter as that of the unexposed controls. The rats exposed to HD and to HD plus A had smaller pupils at the end of the post-exposure period compared to the controls (Table 1).

The penetration of n-hexane metabolites through the blood-aqueous humor/retina barriers is described in Paper VI. Albino rats were exposed to a low dose of 2,5-HD by tube feeding for 35 days and then kept for 28 days in the same environment with no the exposure 2,5-HD. Other albino rats were exposed to a high dose of 2,5-HD for 11 days and allowed post exposure periods of 2 and 4 weeks. Only the high dose rats had serious neurological signs e.g. paralysis. The rats were studied morphologically by counting the nuclei of the outer nuclear layer (ONL). Rats exposed to the lower dose of 2,5-HD had the same amount of nuclei immediately after the end of exposure and the post-exposure period. The rats exposed to the higher dose of 2,5-HD had the same amount of nuclei as the rats from the lower dose group immediately after exposure, but a significantly larger loss of nuclei after the post-exposure periods. The retina, aqueous humor, and blood serum were analysed using gas chromatography 6 hours after exposure. The concentration of 2,5-HD in the aqueous humor was about twice that found serum in the low exposed rat. In the high dose group the corresponding difference was about 6 folded.

In an elimination study, albino rats were also exposed to 2,5-HD (0.04 g/kg) at different occasions during 24 hours. The elimination from retina, aqueous humor, and serum were similar. The concentration of 2,5-HD reached the maximum after about 1 hour, almost no 2,5-HD was detected after 24 hours.

7. Discussion

In the present study the neurotoxicological effects of the two n-hexane metabolites, 2,5-hexanedione (2,5-HD) or 2,5-hexanediol (2,5-HDol), were investigated. Affected regions were found in both the central and peripheral nervous systems. The metabolites produce swellings proximal to the nodes of Ranvier at the distal ends of long nerve axons in accordance with previous findings (Cavanagh & Bennetts, 1981; Spencer et al., 1980). The use of immuno-histochemical techniques with monoclonal antibodies to neurofilament protein (NFP) was found to be a simple and straightforward method to identify the swellings caused by exposure to n-hexane metabolites. This histological method showed a better reproducibility than the traditional silver technique, that also stains the connective tissue. It was thus possible to demonstrate that the swellings contained an excess of NFP and not tubulin. Tubulin is, in conformity with neurofilament, a cytoskeletal material and antibodies to the tubulin complex were not visible in the swellings. It was also noticed that most nerves innervating internal organs had swellings. This observation contradicts the previous view that only the large wide axons with Schwann's cells contain swellings. By immuno-histochemical techniques it was also easy to observe that the swellings of the axons were reversible when exposure to n-hexane was ceased.

Different hypothesis have been proposed, concerning how the axonal swellings arise. Some studies suggest that the swellings start when the metabolite of n-hexane, 2,5-HD, hastens the axonal transport leading to an accumulation of neurofilaments at the nodes of Ranvier (Monaco et al., 1990).

It was long thought that the swellings in the nerves innervating the muscles in the limbs cause the paralysis observed after exposure to 2,5-HD or 2,5-Hdol. The mechanism of the neuropathy is, however, still unknown. A symposium was arranged in 1997 by the journal NeuroToxicology in order to discuss the mechanisms underlying the nerve damage causing e.g. paralysis after exposure to n-hexane or its metabolites. The participants at the symposium concluded that the swellings *per se* could not cause the damage of the nerves but that pyrrol products formed by an interaction between neurofilament lysine residues and 2,5-HD could cause the paralysis. In the axons, the neurofilament protein lysine ε-amino residues are suggested to react with 2,5-HD to form pyrrol substances (Graham et al., 1982). Initial pyrrol formation is followed by autoxidation and a subsequent nucleophilic attack leading to covalent intermolecular NF-NF crosslinkings and disruption of the cytoskeleton (Graham, 1997). Progressive proximo-distal covalent derivation promotes NF-accumulation and subsequent swelling at distal paranodal regions where the normal narrowing of the axon impedes translocation of cross-linked NFs (Graham et al., 1995; Hirai et al., 1999a). In support of the pyrrol hypothesis, pyrrolated cytoskeletal proteins have been demonstrated in

central and peripheral nervous tissues from γ-diketone-intoxicated animals (Ogata et al., 1991). It has been shown (Sickles et al., 1994) that in crayfish lacking NF-proteins, axonal degeneration may occur. This suggests that axonal NF-proteins are not obligatory for the induction of γ-diketone neurotoxicity. The intoxication thus seem to produce atrophy virtually in the absence of swellings. Hirai et al. opposed this result and found that neurofilament deficient quail does not suffer from chronic degeneration on the nervous system (Hirai et al., 1999b). Also other reactions between HD and proteins leading to the formation of toxic products are conceivable. However, since it is known that some pyrrol molecules are indeed very toxic, it is likely that the pyrrol substances formed from neurofilament lysine residues and HD play an important role in the processes leading to paralysis.

There are two main theories related to the damage of the nerves: (i) as a consequence of impeded transport of nourishment from the cell body there is a *degeneration* of the nerve fibres distal to the node of Ranvier, or (ii) as a result of a reaction between 2,5-HD and axonal proteins, a toxic substance is produced and causing *atrophy* of the nerve cytoskeleton. Little is known about the pyrrol products but for example dimethyl pyrrol is a very reactive and toxic substance. Pyrrol products have been observed in the swellings, blood and urine.

It has been suggested that the serious weight loss which occurs when animals are exposed to 2,5-HD or 2,5-HDol may produce the axonopathy due to a reduction of energy production (Medrano & LoPachin, 1989). The authors also showed that the hexane metabolites in all brain regions produce a substrate-dependent inhibition of brain mitochondrial respiration. A combination of degeneration caused by deficiency of nourishment and atrophy by formation of a very toxic chemical seemed to be a possible reason.

Severe effects of n-hexane metabolites have been observed also in non-nervous tissue. Testicular atrophy was seen after exposure to 2,5-HD but no axonal alterations were detected (Boekelheide, 1987). There was destruction of the Sertoli cells and a considerable loss of spermatozoan germ cells and consequently of the germ cells. It is likely that 2,5-HD had a direct toxic effect on the testes. Following the 2,5-HDol induced loss of spermatozoan cells, germ cells were seen to return during the post-exposure period. This implies that a sufficient number of mother cells were still left at the end of the exposure period (Nylén et al., 1989).

There were also distinct dermatological effects of 2,5-HD exposure. The skin was coloured yellow and had brown flakes. The 2,5-HD is suggested to enter the skin cells from inside, have a direct toxic effect on the skin epithelial cells. It is, however, also possible that this toxic effect is a consequence of a phototoxic reaction caused by the combined exposure to light and 2,5-HD.

The following studies (II - VI) became focused on damage to the eyes by n-hexane metabolites. The exposed rats in Paper II had lost half of the photoreceptor cells immediately after the end of exposure, and after the recovery period there were few photoreceptors left in the retina. In contrast, at ora serrata, an area with sparse light exposure, the photoreceptor cells were nearly intact. This observation

indicates that besides 2,5-HD, also light energy may contribute to the retinal alterations. A histological investigation showed swollen axons in iris tissue. This observation suggests that neuronal damage causes a more open pupil, resulting in an increased light exposure contributing to the damage of the retina (Abdel-Rahman et al., 1978). The reduction of the photoreceptor cells might even point to the fact that the pupil had to be more open to properly achieve enough light to activate the retina sufficiently, (Paper V).

Paper III demonstrated a difference between rods and cones following combined exposure to n-hexane metabolites and light energy. It was shown that the rods were more sensitive to 2,5-HD in combination with light energy than the cones. This is probably explained by the fact that rods absorb light from the whole spectrum whereas the cones absorb light in either the blue, green or red regions of the spectrum. The emission spectrum of the standard fluorescent tubes used in the present studies contained sufficient energy at violet-blue wavelengths (paper IV) to cause photoreceptor cell damage in rats (Noell et al., 1966; Sperling et al., 1980). The significant loss of cones seen after the combined exposure to 2,5-HD and light is thus most likely caused by damage to violet-blue absorbing cones. The cones absorbing light of red-yellow wavelengths may have remained more intact.

The results support the hypothesis that there exists a connection between occupational exposure to n-hexane and colour vision alterations. This present results correlates well with the clinical observations (Raitta et al., 1978) that workers exposed to n-hexane had difficulties in discriminating colours along the yellow-blue axis. Their conclusion was that the defect was caused by injuries to the cones absorbing blue light. To put this interpretation into a European perspective, in northern Europe fluorescent tubes usually emit less blue light than in the southern parts (Roger Wibom, personal communication) where tubes emitting considerable more energy in the blue part of the spectrum are preferred. This fact in combination with the more intense sunlight opens up the possibility of a higher risk for light and chemically induced retinal alterations in individuals exposed to n-hexane in the Mediterranean countries.

The study design, including the reduced light exposure, in Paper IV and V was chosen to achieve information whether 2,5-HDol or light had effects of their own. The ONL was reduced about 30 % immediately after 5 week exposure to 2,5-HDol and light whereas no significant retinal damage was observed in the group exposed to 2,5-HDol in darkness or to light alone. Physiological investigation with electroretinogram (ERG) agreed with these results, except for a small tendency of 2,5-HDol treated albino rats immediately after exposure in darkness, where the photoreceptor displayed a certain loss of function which disappeared during the recovery period. This observation was not seen morphologically in Paper IV, and could possibly have been a non-toxic, osmotic effect.

In the presence of light, 2,5-HDol exposure caused damage to the albino rat retina (Paper IV). The reduction in the number of photoreceptors progressed after the termination of the 2,5-HDol exposure. On the other hand, in total darkness

there was no loss of photoreceptor cells in exposed albino rats. This observation further support the notion that both light energy and 2,5-HD are required to cause a substantial damage within the retina of albino rats. There was also a potentiation of the light induced damage during the post-exposure period. In addition, it was demonstrated that the retinal damage caused by 2,5-HDol together with light can exceed a simple summation of the minor action of these two agents alone after the post-exposure periods. The present findings emphasise that long observation times are required in order to perform a reliable morphological detection of photoreceptor loss.

Pigmented rats developed clinically less severe signs of neurological dysfunction than those seen in albinos after exposure to equivalent doses of 2,5-HDol. Whether this reflects some sort of protection, e.g. by the pigment melanin, or a strain specific difference is unclear. Exposed pigmented rats showed, particularly after 13 weeks without 2,5-HDol, a significant loss of photoreceptors whether in light or total darkness. This result was different from the exposed albino rats which had no loss of photoreceptors in total darkness. The pigmentation of the iris in pigmented rats can be expected to attenuate the amount of light reaching the retina (Rapp & Williams, 1980) and hence to reduce the risk of light induced damage to the retina. The results indicate, however, that the 2,5-HDol affects the photoreceptors of the pigmented rats both in light and darkness. There is at present no reasonable explanations to this finding.

Weight loss was seen in all albino and some pigmented rats already from the beginning of the exposure to 2,5-HDol. One reason for the weight loss could be that the hexane metabolites affected the metabolic system, e.g. inhibiting mitochondrial respiration (Medrano & LoPachin, 1989), which together with a lower water and food intake caused a weight loss. However, a general malnutrition as a significant part of the pathogenesis of the ONL cell loss is excluded. During the exposure to 2,5-HDol, animals housed in darkness showed a retarded weight gain similar to those exposed to both 2,5-HDol and light, and with only the latter group clearly exhibiting photoreceptor loss.

Albino rats were exposed to 2,5-HDol in order to decide to what extent the n-hexane metabolites penetrate the blood-aqueous humor/retina barriers. The results from an elimination study and a long term exposure (Paper VI) indicated that 2,5-HD penetrates the blood-aqueous humor/retina barriers and is concentrated within the aqueous humor. This result indicates an active transport of 2,5-HD from serum to the aqueous humor. However, in spite of this acute accumulation no 2,5-HD was detected after 24 hours. The long term exposure thus indicates that there is no long-term accumulation of 2,5-HD. Rats exposed to a lower dose of 2,5-HD had no significant reduction of the number of nuclei in the outer nuclear layer (ONL), neither immediately after the end of exposure nor after the post-exposure periods. The rats exposed to higher doses had, however, a significantly reduced number of nuclei after the postexposure periods. These observations are in accordance with results from earlier studies (paper II) and indicates a dose related toxic reaction.

Mechanisms

The results of the present study may suggest the following mechanism for the light induced toxic effects of 2,5-HD. A key factor in the destruction of the photoreceptors is rhodopsin, which is a compound located in the OS, the laminated structure of the photoreceptor cells. Rhodopsin is a chromophore consisting of the aldehyde *cis*-retinal and the protein opsin, which are combined together through the amino acid ε lysine by means of an Schiff´s base bond. When rhodopsin is exposed to light, *cis*-retinal changes to all *trans*-retinal, which in turn can not bind to the opsin and the two components separate. It is suggested that during exposure to n-hexane metabolites, the all *trans*-retinal is replaced by 2,5-HD. A chemical reaction between 2,5-HD and light energy would then lead to the formation toxic pyrrol substances in a similar fashion as in the motor nerve. The toxic products may either directly damage the retinal cells, or do so via a further interaction with light. There is support for this notion. During intoxication with n-hexane many more pyrrol rings were formed than were oxidised, leaving the pre-formed pyrrolyl derivatives vulnerable to oxidation during the postexposure period (Graham et al., 1995). The severity of the neuropathy continues to progress for one to four months following cessation of exposure. Pyrrolic substances were still present in hair samples 25 days after cessation of 2,5-HD administration (Johnson et al., 1995). Zue et al. demonstrated a free-radical dependence of autoxidative dimerization (Zhu et al., 1994). Alkylpyrrol adducts are susceptible to secondary autoxidative reactions that result in the covalent protein crosslinking that is an obligatory step in 2,5-HD neuropathy.

There are obviously other possible mechanisms. For example, 2,5-HD is a diketone. Ketones are believed to be photosensitizers and thus capable of damaging the photoreceptor cells. A Type II reaction could then occur transferring the oxygen atoms to a higher energy level, to singlet oxygen. Singlet oxygen is a molecule of high energy which can cause e.g. lipid peroxidation and other cytotoxic reactions. However, no previous studies confirming this mechanism have been found. Alternatively, 2,5-HD exposure could affect the levels of light protecting substances like ascorbic acid and other antioxidants in the aqueous humor, and thus result in an increased risk for retinal damage. It is also possible that the synergistic toxic effect of light energy and 2,5-HD on the retina is caused by a reduced activity of mitochondrial cytochrome oxidase. Cytochrome oxidase, which is an important enzyme in the respiratory chain, is a chromophore absorbing photons of a wavelength around 440 nm, i.e. wavelengths corresponding to the spectrum of some fluorescent tubes (paper IV). Indeed, it has been observed that light from fluorescent tubes decreases the activity of cytochrome oxidase in the retina and that the consequence is cellular damage (Chen, 1993; Hansson, 1970; Spencer et al., 1980).

The mechanisms by which n-hexane metabolites make the photoreceptors more susceptible to light in the albino rats is unclear. In a parallel study (Bäckström et

al., unpublished data) the retinal alterations after exposure to two substances with similar effects on the axon, disulfiram and acrylamide, were investigated using albino rats exposed to different light conditions. Exposure to disulfiram, or its metabolite carbon disulphide, and acrylamide caused the same neurological symptoms as with the n-hexane metabolites, i.e. swellings of the axons and paralysis. A morphological investigation of the eyes showed loss of photoreceptors in the disulfiram exposed albino rats. On the other hand, the acrylamide exposed albino rats expressed no loss of photoreceptor cells, neither immediately after the exposure nor following recovery periods in light or darkness. The investigation thus presented no evidence that the toxic mechanisms involve a common pathway for all analogues to cause the well documented cytoskeletal pathologies (Cavanagh, 1982b; Cavanagh & Bennetts, 1981; Spencer et al., 1980).

8. Conclusion

1: The use of monoclonal antibodies against neurofilament results in a better reliability than the traditional silver technique in evaluation of axonal abnormalities induced by 2,5-HD or 2,5-HDol.

2: Swollen axons contain an enhanced amount of neurofilament but not of tubulin.

3: 2,5-HD and 2,5-HDol exposed rats have swollen axons in several major organs.

4: Retinal damage is induced by exposure to 2,5-HD or 2,5-HDol.

5: The presence of light is necessary to produce the 2,5-HD or 2,5-HDol induced retinal damage. In albino rats exposed to 2,5-HDol in total darkness, no loss of photoreceptors is observed.

6: In the presence of light, the retinal damage continues after the end of exposure to 2,5-HD or 2,5-HDol.

7: 2,5-HD penetrate the blood-aqueous humor/retina-barriers and is eliminated within 24 hours.

9. Summary

Birgitta Bäckström: *Structural and functional alterations in the rat retina after long term exposure to two n-hexane metabolites.* Arbete och Hälsa 1999:19, National Institute for Working Life, Stockholm.

Axonal abnormalities induced by exposure to the n-hexane metabolites 2,5-hexanedione (2,5-HD) or 2,5-hexanediol (2,5-Hdol) were studied in rats by immunohistochemical techniques using monoclonal antibodies against neuro-filament proteins. These techniques produced more distinct and reproducible results than the traditional silver technique. It could thus be shown that the swollen axons contained increased amounts of neurofilaments but not of tubulin. In most of the major organs exposed to 2,5-HD or 2,5-HDol swollen axons were observed. The exposed rats showed motor impairment, mainly paralysis. Testicular and retinal degeneration were also seen. In contrast to the retinal effects, the testicular changes and the paralysis disappeared after ending the exposure.

Immediately after the exposure to 2,5-HD or 2,5-Hdol, the retina had a loss of photoreceptor cells that progressed during the post-exposure periods. The cones were more resistant than the rods to the n-hexane metabolites and light exposure. A greater survival of photoreceptors at the ora serrata in exposed albino rats suggested that the 2,5-HD or 2,5-HDol effects require light in order to cause cellular damage. Swollen axons were found in the iris of exposed rats, which might be consistent with an observed impairment of pupil function, and thus an increased retinal light exposure and possibly enhanced photoreceptor loss. Albino rats exposed to 2,5-HDol in total darkness had no loss of photoreceptors.

Electroretinography confirmed the morphological observations of damage to, and loss of, photoreceptor cells in the presence of light from fluorescent tubes when exposed to 2,5-HD or 2,5-HDol. This toxic effect on the retina progressed during the post-exposure periods.

2,5-HD penetrates the blood-aqueous humor/retina barrier. The concentration of 2,5-HD in the aqueous humor was considerably higher than in serum. This indicated an active transport from blood to aqueous humor. Twenty-four hours after the exposure no 2,5-HD could be found, demonstrating that there is no long-term accumulation of 2,5-HD.

Keywords: n-hexane, 2,5-hexanedione, 2,5-hexanediol, neurotoxicity, retinopathy, photoreceptors, light energy, electrophysiology, pupil.

10. Sammanfattning (Summary in Swedish)

Birgitta Bäckström: *Structural and functional alterations in the rat retina after long term exposure to two n-hexane metabolites.* Arbete och Hälsa 1999:19, National Institute for Working Life, Stockholm.

Exponering för n-hexanmetaboliterna, 2,5-hexandion (2,5-HD) och 2,5-hexandiol (2,5-HDol) medförde svällda axoner i flera av råttans olika organ. Även testikel och retinaskador påvisades. Märkning med monoklonala antikroppar mot neurofilamentprotein och tubulin visade att de svällda axonerna innehåller ökad mängd neurofilament men ej tubulin. Immunperoxidastekniken ger en snabb, precis visualisering av axonerna och dessas eventuella skador.

Förlamning och testikelskador gick tillbaka efter avslutad exponering för n-hexanmetaboliterna. Fotoreceptorskadorna förvärrades emellertid även efter avslutad exponering. Kvarvarande fotoreceptorer vid Ora Serrata visar att 2,5-HD och 2,5-HDol är beroende av ljusenergi för sin skadande effekt på näthinnan. Tapparna, som utgör 2-3 % av fotoreceptorerna, tycks mer resistenta mot exponering för n-hexanmetaboliter och ljus än stavarna.

Ingen retinal skada vid exponering för 2,5-HD kunde påvisas morfologiskt hos albinoråttor i totalt mörker. Däremot kunde man konstatera att 2,5-HD potentierar ljusinducerad morfologisk skada, som fortsätter efter upphörande av exponeringen för n-hexanmetaboliterna. En elektrofysiologisk ögonundersökning bekräftade de morfologiska resultaten.

2,5-HD penetrerade blod-ögonkammar/näthinnebarriärerna. 2,5-HD koncentreras därvid i ögonkammarvätskan, sannolikt mot en koncentrations-gradient. Ingen ackumulation av 2,5-HD under långtidsexponering som kunde förklara den fortsatta förlusten av fotoreceptorer efter avslutad exponering kunde påvisas. De uppkomna toxiska skadorna var dosrelaterade.

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