

Formate-Induced Inhibition of Photoreceptor Function in Methanol Intoxication¹

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Accepted for publication December 11, 1998 This paper is available online at <http://www.jpet.org>

ABSTRACT

Formic acid is the toxic metabolite responsible for the retinal and optic nerve toxicity produced in methanol intoxication. Previous studies in our laboratory have documented formate-induced retinal dysfunction and histopathology in a rodent model of methanol intoxication. The present studies define the time and concentration dependence of formate-induced retinal toxicity in methanol-intoxicated rats. Retinal function was assessed 24, 48, and 72 h after the initial dose of methanol by flicker electroretinographic measurements. Retinal histopathology was assessed at the same time intervals. Rod- and cone-mediated electroretinogram (ERG) responses were attenuated in a formate concentration- and time-dependent manner, and both retinal sensitivity and maximal responsiveness to light were diminished. Attenuation of UV-cone-mediated responses was temporally delayed in comparison to the functional deficits observed in the 15 Hz/510 nm responses, which have a rod-

mediated component and occurred at significantly higher formate concentrations. Both 15 Hz/510 nm and UV-cone-mediated ERG responses were undetectable by 72 h; however, if light intensity was increased, a retinal ERG response could be recorded, indicating that photoreceptor function was profoundly attenuated, but not abolished, under these intoxication conditions. Functional changes preceded structural alterations. Histopathological changes were most pronounced in the outer retina with evidence of inner segment swelling, photoreceptor mitochondrial disruption, and the appearance of fragmented photoreceptor nuclei in the outer nuclear layer. The nature of both the functional and structural alterations observed are consistent with formate-induced inhibition of mitochondrial energy production, resulting in photoreceptor dysfunction and pathology.

Methanol is an important public health and environmental concern because of the selective actions of its neurotoxic metabolite, formic acid, on the retina and optic nerve. Both acute and chronic methanol exposure have been shown to produce retinal dysfunction and optic nerve damage clinically (Sharpe et al., 1982; Kavet and Nauss, 1990; Eells, 1992) and in experimental animal models (Ingemansson, 1983; Eells, 1991; Murray et al., 1991; Lee et al., 1994). Methanol is commonly used as an industrial organic solvent and is available to the public in a variety of products. It is also being developed as an alternative fuel and energy source (Kavet and Nauss, 1990). The expanded use and availability of methanol increases the probability of accidental acute or chronic methanol exposure and underscores the importance of understanding the mechanisms responsible for its toxicity.

Humans and nonhuman primates are uniquely sensitive to

the toxic effects of methanol (Hayreh et al., 1980; Kavet and Nauss, 1990; Eells, 1992). Methanol poisoning in humans and monkeys is characterized by an initial mild central nervous system depression, followed by an asymptomatic latent period lasting about 12–24 h. The latent period is followed by a syndrome consisting of formic acidemia, uncompensated metabolic acidosis, visual toxicity, coma, and in extreme cases, death. Initial signs of visual toxicity include misty or cloudy vision, and ophthalmoscopic examination typically reveals retinal and optic disc edema.

Formic acid is the toxic metabolite responsible for the metabolic acidosis and ocular toxicity observed in human methanol poisoning (Hayreh et al., 1980; Kavet and Nauss, 1990; Eells, 1992). Nonprimate species are ordinarily resistant to the accumulation of formate and the associated metabolic and visual toxicity (Eells et al., 1981; Eells, 1992). Our laboratory has developed a nonprimate model of methanol toxicity using rats in which formate oxidation has been selectively inhibited by treatment with nitrous oxide (Eells et al., 1981, 1996b; Eells, 1991; Murray et al., 1991). Subanesthetic concentrations of nitrous oxide inactivate the enzyme methionine synthase, reducing the production of tetrahydro-

Received for publication March 3, 1998.

¹ The project described was supported by Grant ES06648 from the National Institute of Environmental Health Sciences, National Institutes of Health, and by Grant EY01931 from the National Eye Institute, National Institutes of Health. This research was presented in part at the 1997 Annual Meeting of the Association for Research in Vision and Ophthalmology, Fort Lauderdale, FL.

ABBREVIATIONS: ERG, electroretinogram; LRRl, log relative retinal illumination.

folate, the cosubstrate for formate oxidation, thus allowing formate to accumulate to toxic concentrations after methanol administration (Eells et al., 1981). Methanol-intoxicated rats developed formic acidemia, metabolic acidosis, and visual toxicity analogous to the human methanol poisoning syndrome. Studies in our laboratory have established this rodent model of methanol-induced visual toxicity and have documented abnormalities in both the flash-evoked cortical potential and the electroretinogram (ERG), as well as histopathologic changes in the neural retina and the optic nerve in methanol-intoxicated rats (Eells et al., 1981, 1996b; Eells, 1991; Murray et al., 1991).

Formic acid has been hypothesized to produce retinal and optic nerve toxicity by disrupting mitochondrial energy production (Hayreh et al., 1980; Sharpe et al., 1982). Formic acid has been shown *in vitro* to inhibit the activity of cytochrome oxidase, a vital component of the mitochondrial electron transport chain involved in ATP synthesis (Nicholls, 1975). Inhibition occurs subsequent to the binding of formic acid with the ferric heme iron of cytochrome oxidase, and the apparent inhibition constant is between 5 and 30 mM (Nicholls, 1975). Concentrations of formate present in the blood and tissues of methanol-intoxicated humans, nonhuman primates, and rodent models of methanol intoxication are within this range (Hayreh et al., 1980; Sejersted et al., 1983; Eells, 1991).

Many questions remain with respect to the time and concentration dependence of formate-induced retinal and optic nerve toxicity, the nature of the functional deficits produced, and the differential sensitivity of retinal cell types to the cytotoxic actions of formic acid. The present studies were undertaken to define the nature of the functional and structural alterations produced in the retinas of rats exposed to steadily increasing concentrations of formic acid after methanol administration. These studies determined the intensity-response relationships of photoreceptor-mediated responses at discrete times during methanol intoxication. Functional deficits were correlated with histopathologic alterations at the same intervals. These studies provide evidence that the absolute sensitivity of both rod- and cone-mediated responses is attenuated in methanol-intoxicated rats in a formate concentration- and time-dependent manner. Attenuation of cone sensitivity was temporally delayed in comparison with the functional deficits in ERGs with a rod component and occurred at significantly higher formate concentrations. Moreover, the nature of the functional and structural changes produced in the retina are consistent with the hypothesis that formate inhibits photoreceptor mitochondrial energy production, resulting in photoreceptor dysfunction and damage.

Experimental Procedures

Materials

Methanol (HPLC grade) obtained from Sigma Chemical Co. (St. Louis, MO) was diluted in sterile saline and administered as a 20% w/v solution. Thiobutabarbital sodium salt (Inactin) was purchased from Research Biochemicals International (Natick, MA). Atropine sulfate was obtained from AmVet Pharmaceuticals (Fort Collins, CO). Hydroxypropyl methylcellulose (2.5%) drops were acquired from IOLAB Pharmaceuticals (Claremont, CA). Atropine sulfate ophthalmic solution drops were purchased from Phoenix Pharmaceutical, Inc. (St. Joseph, MO). All other chemicals were reagent grade or better.

Animals

Male Long-Evans rats (Harlan Sprague-Dawley, Madison, WI), which weighed 250 to 350 g, were used throughout these experiments. All animals were supplied food and water *ad libitum* and maintained on a 12-h light/dark schedule in a temperature- and humidity-controlled environment. Animals were handled in accordance with the Declaration of Helsinki and/or with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health.

Methanol-Intoxication Protocol

Rats were placed in a Plexiglas chamber (22 × 55 × 22-cm) and exposed to a mixture of N₂O/O₂ (1:1; flow rate, 2 liters/min) for 4 h before the administration of methanol or saline. N₂O/O₂ exposure was continued throughout the course of the experiment. Methanol (20% w/v methanol in saline) was administered by *i.p.* injection at a dose of 4 g/kg, followed by 2 g/kg supplemental doses every 24 h after the initial dose. Controls for these experiments included groups of rats treated with saline and exposed to: nitrous oxide (N₂O-control); rats treated with methanol, but not exposed to nitrous oxide (methanol-control); and untreated rats (untreated-control). Rats were periodically removed from the exposure chamber for electrophysiological measurements and to obtain blood samples. Blood samples for formate analysis were obtained from the tail vein. Formate concentrations were determined from blood samples as described previously (Eells, 1991). Rats were euthanized by an overdose of pentobarbital, followed by decapitation.

ERG Procedures and Analysis

ERG experiments were performed on adult male Long-Evans rats using slight modifications of published procedures (Neitz and Jacobs, 1986). The light stimulation apparatus consisted of a three-beam optical system (Neitz and Jacobs, 1986). All three beams were derived from tungsten-halide lamps (50W, 12V). The intensity of these beams was controlled by using neutral density step filters. All three lamps were underrun at 11 V from a regulated DC power supply. Each of the beams contained a high-speed, computer-driven shutter (Uniblitz, Vincent Associates). One beam had a wavelength computer controlled by a Varispec tunable bandpass filter (Cambridge Research Instruments; half-energy passband, 7 nm). The second beam was used for achromatic light-evoked ERGs under conditions where a bright light was required to elicit an ERG response. The second beam was also used with a short pass UV-filter (half pass, 380 nm) in experiments designed to isolate UV-cones. The third beam was used as a chromatic adapting light to suppress responses from rods and M-cones in the UV-cone isolation experiments. For this purpose, a glass long wavelength pass filter (halfpass, 590 nm) was used. The three beams were optically superimposed and focused on the lens to illuminate a 70° patch of retina in Maxwellian view. Light calibrations were made with a PIN 10 DF silicon photodiode (United Detector Technology).

ERG recordings were differentially amplified and computer averaged. The ERG was differentially amplified in two stages. The first amplifier was positioned near the recording electrode, and the second was within the signal processing system. The signal processing system was implemented as a custom-made, single plug-in board in a PC. The amplified signal was processed through a two-stage active narrow bandpass filter (the half voltage of this filter was 0.2 times the center frequency). The filter has a center frequency set equal to the modulation frequency of the test light. To ensure that any transients in the response that occur at the onset of the stimulus pulses were not included in the average, the initiation of signal averaging was delayed by a preset number of stimulus cycles (typically a minimum of 20). The resulting ERG is an extremely noise-free, single cycle, sinusoidal wave form. The averaged responses were measured (peak-to-trough amplitude) from a calibrated digital oscilloscope display (Neitz and Jacobs, 1986).

Before ERG analysis, ophthalmoscopic examination confirmed that all eyes were free of lenticular opacities or other gross anomalies. Rats were anesthetized with thiobutabarbital sodium salt (100 mg/kg, i.p.), positioned in a Kopf stereotaxic apparatus, and placed on a heating pad to maintain core body temperature at 37°C. Atropine sulfate (0.05 mg/kg, s.c.) was administered to inhibit respiratory tract secretions. The pupil of the eye to be tested was dilated by topical application of 1% atropine sulfate. Methylcellulose was topically applied as a lubricant and to enhance electrical conduction. A circular silver wire recording electrode was positioned on the cornea, a reference electrode was placed above the eye, and a ground electrode was placed on the tongue. Flicker ERG analyses were performed at 24, 48, and 72 h after the initial dose of methanol to define the effects of formic acid accumulation on retinal function at discrete times during methanol intoxication. Rats were not dark adapted, and all recordings were obtained under ambient light conditions from cool white fluorescent room lights ~ 100 cd/m² at the rat's eye. Flickering stimuli (light/dark ratio, 0.25:0.75) were presented. Responses to 60 successive flashes were averaged for each stimulus condition. At each test wavelength, a minimum number of four stimulus intensities, spaced at intervals of 0.3 log unit, were presented. All sensitivity measures were made in triplicate.

Three experimental protocols were used to evaluate retinal function.

Protocol 1: 15 Hz/510 nm ERG Response. ERGs were recorded in response to a 15-Hz flickering light at a wavelength of 510 nm over a 3-log unit range of light intensity. For these studies, the unattenuated stimulus (log relative retinal illumination, 0) had an irradiance of 25 μ W distributed over the 70° patch of illuminated retina. This can be calculated to produce retinal illumination equivalent to about 10⁴ scotopic trolands. These recording conditions are disadvantageous to rods; however, because at least 97% of rat photoreceptors are rods and ERGs are recorded at luminance intensities ranging from 10¹ to 10⁴ scotopic trolands, it is likely that the responses to the 15 Hz/510 nm light are drawn from both rods and medium wavelength cones (M-cones) (Jacobs and Neitz, 1989; Szel and Rohlich, 1992; Deegan and Jacobs, 1993).

Protocol 2: UV-Cone-Mediated ERG Response. Cone responses were elicited by a 25-Hz flickering ultraviolet light (380-nm cut off) in the presence of an intense chromatic adapting light (445 μ W), which eliminated responses mediated by rods and M-cones (Jacobs et al., 1991). Recording conditions were the same as those used by Jacobs et al. (1991). In this report, complete spectral sensitivity functions were measured in the rat, and it was demonstrated that UV-cone responses were separated from rod and M-cone responses. UV-cone-mediated ERG responses were recorded over a 1-log unit range of light intensity. For these studies, the unattenuated stimulus (log relative retinal illumination, 0) had an irradiance of 12.5 μ W distributed over the 70° patch of illuminated retina. By equating the effectiveness of this light to the 510-nm stimulus, we estimate that the unattenuated light produced the equivalent of 10^{2.5} scotopic trolands in the rat eye.

Protocol 3: Achromatic Light Response. Preliminary experiments indicated that severely intoxicated animals did not respond to the most intense illumination presented at 510 nm. Therefore, a third protocol was used to provide a measure of the degree of loss of retinal function in severely intoxicated animals. These experiments measured the intensity of an achromatic light stimulus (15 Hz) required to elicit a criterion 5 μ V ERG response (achromatic light response). Achromatic light responses were recorded over a 3-log unit range of light intensity. For these studies, the unattenuated stimulus (log relative retinal illumination, 0) had an irradiance of 8 mW distributed over the 70° patch of illuminated retina. By equating the effectiveness of this light to the 510-nm stimulus, we estimate that the unattenuated light produced the equivalent of 10⁶ scotopic trolands in the rat eye.

Histopathologic Analysis

Animals were anesthetized with sodium pentobarbital (60 mg/kg) and decapitated. Eyes were enucleated, hemisected, and immersed in fixative (2.5% paraformaldehyde, 1.5% glutaraldehyde, and 4% sucrose in 0.1 M phosphate buffer at 4°C, pH 7.2) for 72 h. The anterior segment and vitreous were removed, then full-thickness pieces of eye wall were dissected from the posterior pole, including the optic nerve. Tissues were postfixed in phosphate-buffered 2% osmium tetroxide (OsO₄), dehydrated in a graded ethanol series, and embedded in epoxy resin. Thick sections (1 μ m) for light microscopy were stained with toluidine blue; thin sections for electron microscopy were stained for uranyl acetate-lead citrate (Murray et al., 1991).

Statistical Analysis

Statistical analysis of group means were made using a group Student's *t* test if only one comparison was made between two groups. In all cases in which several comparisons were required, one-way ANOVA with repeated measures was performed. This was followed by a Dunnett's test procedure for multiple comparisons with a control (Winer, 1972). In all cases, the minimum level of significance was taken as *P* < .05.

Results

Development of Formic Acidemia in Methanol-Intoxicated Rats

The administration of methanol to N₂O-exposed rats has been shown to result in the accumulation of formate in the blood as a consequence of the inhibition of formate oxidation (Eells et al., 1981, 1996b; Eells, 1991; Murray et al., 1991). In the present studies, methanol was administered at an initial dose of 4 g/kg, followed by supplemental doses of 2 g/kg at 24-h intervals. As shown in Fig. 1, rats treated with methanol in the presence of N₂O accumulated increasing concentrations of formate over the course of the experiment. Blood formate concentrations in methanol-intoxicated rats increased from basal, endogenous concentrations of 0.8 \pm 0.1 mM to 2.6 \pm 0.2 mM, 24 h after the initial dose of methanol. Forty-eight h after the initial dose of methanol, blood formate concentrations had increased to 4.8 \pm 0.3 mM, and by 72 h, blood formate concentrations in methanol-intoxicated rats were 8.0 \pm 0.4 mM. Blood formate concentrations measured in N₂O-control (0.9 \pm 0.2 mM) and methanol-control rats (1.0 \pm 0.3 mM) were not significantly different from blood formate concentrations measured in untreated-control rats (0.8 \pm 0.1 mM).

Disruption of Retinal Function in Methanol-Intoxicated Rats

The objectives of the present studies were: 1) to analyze the loss of outer retinal function in animals exposed to steadily increasing concentrations of formic acid after methanol intoxication; and 2) to examine the effects of methanol intoxication on photoreceptor function. To accomplish these objectives, we made some methodological modifications and compromises in our experimental design. Previous studies in our laboratory and by other investigators have assessed formate-induced retinal dysfunction after methanol intoxication by measurement of flash-evoked ERG responses at a single light intensity in dark-adapted animals (Eells, 1991, 1996b; Murray et al., 1991; Lee et al., 1994). In the present studies, a flicker electroretinography approach was used to enable a

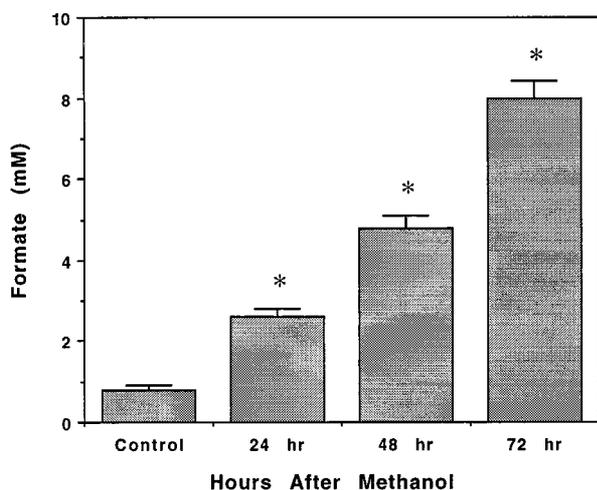


Fig. 1. Blood formate concentrations in methanol-intoxicated and untreated-control rats. Methanol-intoxicated rats were exposed to a mixture of N_2O/O_2 (1:1) for 4 h before methanol administration (4 g/kg at zero time, followed by 2 g/kg at 24-h intervals), and exposure to the gas mixture was continued throughout the experiment. Blood formate concentrations were determined before methanol administration and at 24-h intervals after methanol administration for 72 h. Shown are the mean values \pm S.E.M. from at least six rats in each experimental treatment group. Significant differences from zero time measurements are indicated with an asterisk (Student's *t* test; $P < .05$). Blood formate concentrations in methanol-control [exposed to room air and administered methanol (4 g/kg at zero time, followed by 2 g/kg at 24-h intervals)] and N_2O -control rats [exposed to N_2O/O_2 (1:1) and administered saline on the same injection schedule] did not exceed 1.0 mM (data not shown).

more accurate and detailed analysis of the loss of outer retinal function over the course of methanol intoxication. Flicker ERG responses rather than single-flash ERG responses were determined in the present studies for the following reasons: 1) under our experimental conditions, flicker responses provided a more sensitive and reproducible assessment of retinal function than single flash measurements; and 2) flicker measurements enabled us to conduct more detailed studies on each animal over a greater range of luminance intensities than would have been feasible using a single-flash protocol. In our studies, the experimental animals are compromised by the neurotoxic actions of methanol, and maintenance of an appropriate level of anesthesia during dark adaptation and ERG measurements is extremely difficult. For this reason, all ERG measurements were conducted under ambient light conditions. Flicker ERG analyses were performed at 24, 48, and 72 h after the initial dose of methanol to define the effects of formic acid accumulation on retinal function at discrete times during methanol intoxication.

15 Hz/510 nm ERG Response. During preliminary experiments, we determined that a 15 Hz/510 nm light produced a robust and reproducible ERG response, illustrated by the control tracing in Fig. 2. These recording conditions are disadvantageous to rods; however, because at least 97% of rat photoreceptors are rods and ERGs are recorded at luminance intensities ranging from 10^1 to 10^4 scotopic trolands, it is likely that the responses to the 15 Hz/510 nm light are drawn from both rods and medium wavelength cones (M-cones) (LaVail, 1976; Jacobs and Neitz, 1989; Szel and Rohlich, 1992; Deegan and Jacobs, 1993). Moreover, it is likely that ERG responses recorded at the lower luminance intensities ($<10^3$ scotopic trolands) reflect responses with a

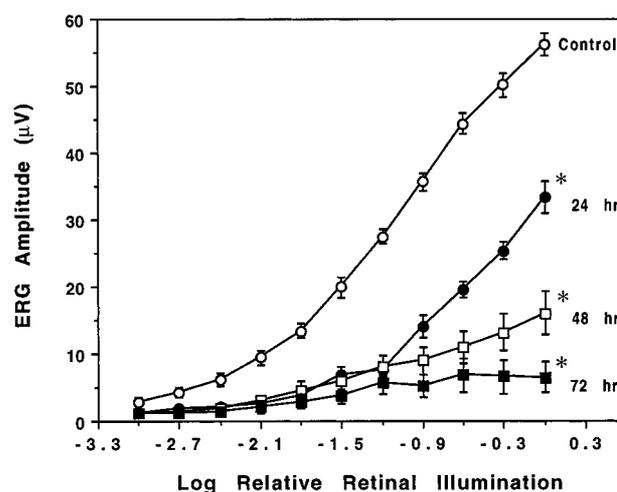


Fig. 2. 15 Hz/510 nm ERG responses in methanol-intoxicated and untreated-control rats. ERGs were recorded from anesthetized rats in response to a 15 Hz flickering light over a 3-log unit range of light intensity at a wavelength of 510 nm. For these studies, the unattenuated stimulus (log relative retinal illumination, 0) had an irradiance of $25 \mu W$ distributed over the 70° patch of illuminated retina. This can be calculated to produce retinal illumination equivalent to $\sim 10^4$ scotopic trolands. Flicker ERG analyses were performed at 24, 48, and 72 h after the initial dose of methanol. Shown are the mean values \pm S.E.M. from four to eight rats in each treatment group. Significant differences from values obtained in untreated-control rats are denoted with asterisks (repeated measurement ANOVA with Dunnett's test; $P < .05$).

robust rod component, whereas those responses recorded at the higher luminance intensities ($>10^3$ scotopic trolands) may be dominated by the activity of M-cones. We base this interpretation on flicker photometry studies in the gerbil, which show a shift from rods to cones at 10^3 scotopic trolands (Jacobs and Neitz, 1989).

The effect of methanol intoxication on 15 Hz/510 nm ERG responses is shown in Fig. 2. In untreated-control animals, ERG amplitude increased linearly over the 3-log unit range of retinal illumination intensities, achieving a maximal amplitude of $56.2 \pm 1.6 \mu V$ at maximal retinal illumination [0 log relative retinal illumination (LRR) equivalent to 10^4 scotopic trolands]. A consistent $5 \mu V$ threshold, 15 Hz ERG response was obtained in control animals at -2.4 ± 0.1 LRR. Two primary effects of methanol intoxication on 15 Hz/510 nm ERG responses are apparent in Fig. 2: 1) the amplitudes of the flicker ERG responses were significantly attenuated at all luminance intensities in a formate concentration- and time-dependent manner; and 2) the light intensity required to elicit a threshold ($5 \mu V$) 15 Hz/510 nm ERG response was significantly increased in a formate concentration- and time-dependent manner. A significant elevation in the ERG threshold coupled with an attenuation of ERG amplitude was apparent as early as 24 h after the initial dose of methanol, at a mean blood formate concentration of 2.6 ± 0.2 mM. In 24-h intoxicated animals, the ERG threshold was increased 0.9 log unit from a control value of -2.4 ± 0.1 LRR to -1.5 ± 0.1 LRR. The maximal ERG amplitude response to light stimulation was also attenuated 24 h after the initial dose of methanol from a control value of $56.2 \pm 1.6 \mu V$ to $33.3 \pm 2.4 \mu V$. After 48 h of methanol intoxication, the 15 Hz/510 nm ERG threshold was increased 1.0 log unit, and the maximal response to light stimulation was attenuated to $16.1 \pm 2.9 \mu V$. The mean blood formate concentration in

these animals was 4.8 ± 0.3 mM. In animals intoxicated for 72 h, ERG responses over the entire range of luminance intensities used to assess 15 Hz/510 nm retinal function were at or below the $5 \mu\text{V}$ criterion established for a threshold response. The mean blood formate concentration in these animals was 8.0 ± 0.4 mM. Inability to achieve threshold response after 72 h of methanol intoxication is indicative of a severe deficit in retinal function. No significant differences from values obtained in untreated-control rats were observed in the 15 Hz/510 nm luminance-response relationships measured in N_2O -control (threshold, -2.4 ± 0.1 LRRI; maximal response, $50.7 \pm 7.1 \mu\text{V}$) or methanol-control animals (threshold, -2.6 ± 0.2 LRRI; maximal response, $58.7 \pm 5.1 \mu\text{V}$).

UV-Cone-Mediated ERG Response. The function of UV-sensitive cones was examined by recording the retinal response to a 25 Hz flickering ultraviolet light (380-nm cut off) in the presence of an intense chromatic adapting light. These conditions have been shown to isolate the UV-cone response in the rat retina (Jacobs et al., 1991). Because the spectral sensitivity of the UV-sensitive cone is distinct from that of rods and M-cones, we can be confident that the UV-cone-isolated conditions yield a pure cone response. Figure 3 illustrates the effect of methanol intoxication on the function of UV-sensitive cones in the rat retina. In untreated-control animals, UV-cone-mediated ERG amplitude increased from a minimal value of $2.1 \pm 0.3 \mu\text{V}$ to a maximal value of $15.1 \pm 1.1 \mu\text{V}$ over the log unit range of retinal illumination used in these studies. A $5 \mu\text{V}$ threshold criterion response was obtained in control animals at a LRRI value of -0.56 ± 0.03 .

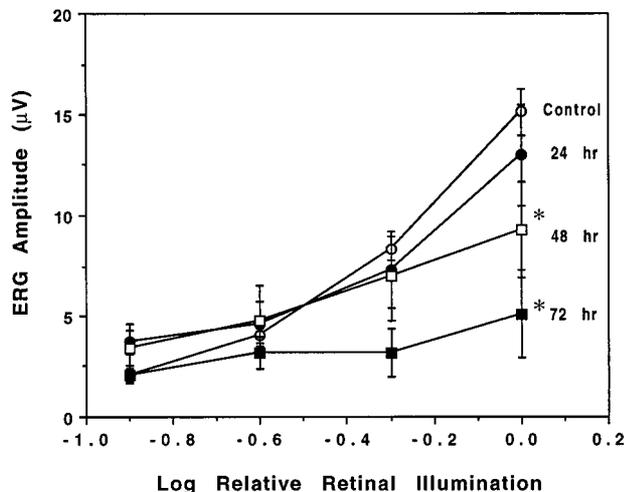


Fig. 3. UV-cone-mediated ERG responses in methanol-intoxicated and untreated-control rats. UV-sensitive, cone-mediated ERGs were recorded from anesthetized rats. Cone responses were elicited by a 25-Hz flickering ultraviolet light (380-nm cut off) in the presence of an intense chromatic adapting light (445 μW), which eliminated responses mediated by rods and M-cones. UV-cone-mediated ERG responses were recorded over a 1-log unit range of light intensity. For these studies, the unattenuated stimulus (log relative retinal illumination, 0) had an irradiance of 12.5 μW distributed over the 70° patch of illuminated retina. By equating the effectiveness of this light to the 510-nm stimulus, we estimate that the unattenuated light produced the equivalent of $10^{2.5}$ scotopic trolands in the rat eye. ERG analyses were performed at 24, 48, and 72 h after the initial dose of methanol. UV-sensitive cone ERGs were recorded from the same animals in which 15 Hz/510 nm ERG responses were recorded. Shown are the mean values \pm S.E.M. from four to eight rats in each treatment group. Significant differences from values obtained in untreated-control rats are denoted with asterisks (repeated measurement ANOVA with Dunnett's test; $P < .05$).

We attribute the smaller dynamic range of the response observed in these studies to the very small percentage of UV-sensitive cones (0.05% of all photoreceptor cells) present in the rat retina (Szel and Rohlich, 1992) and to the lower retinal illuminance produced by the unattenuated UV-light ($10^{2.5}$ scotopic trolands) compared with the unattenuated light at 510 nm (10^4 scotopic trolands).

Similar to the effects observed in 15 Hz/510 nm ERG responses, methanol intoxication also attenuated the amplitude and reduced the absolute sensitivity of UV-cone-mediated ERG responses in a formate concentration- and time-dependent manner. However, reduction of cone-mediated responses was temporally delayed and occurred at significantly higher blood formate concentrations, compared with the functional deficits observed in experiments measuring 15 Hz/510 nm responses. For UV-cone-mediated responses, a significant elevation in the threshold required to elicit a $5 \mu\text{V}$ response was not apparent until 72 h after the initial dose of methanol at a mean blood formate concentration of 8.0 ± 0.4 mM. In rats intoxicated for 24 h, no differences from control values for ERG threshold or ERG amplitudes were apparent in UV-cone-mediated responses. After 48 h of methanol intoxication, the maximal response to UV light stimulation was significantly reduced from a control amplitude of $15.1 \pm 1.1 \mu\text{V}$ to $9.3 \pm 2.4 \mu\text{V}$. In 72-h intoxicated animals, the light intensity required to elicit a $5 \mu\text{V}$ response was not achieved within the range of retinal illumination tested. These findings are similar to those observed with 15 Hz/510 nm ERG responses and are indicative of a severe deficit in cone-mediated retinal function. No significant differences from values obtained in untreated-control rats were observed in the UV-cone-mediated luminance-response relationships measured in N_2O -control (UV-cone ERG threshold, -0.54 ± 0.06 LRRI; maximal response, $14.0 \pm 2.0 \mu\text{V}$) or methanol-control animals (UV-cone ERG threshold, -0.52 ± 0.13 LRRI; maximal response, $12.2 \pm 1.6 \mu\text{V}$).

Achromatic Light Response. The results from experiments using spectral light to assess retinal and UV-cone function showed an inability to achieve threshold response after 72 h of methanol intoxication. Spectral light experiments are limited in maximal light intensity and cannot be used to assess retinal function in animals with very high threshold elevation. Therefore, after the differential assessment of 15 Hz/510 nm and UV-cone-mediated function, a final ERG experiment was performed to assess the degree of loss of outer retinal function in intoxicated animals. This experiment measured the intensity of an achromatic light stimulus (15 Hz, 10^3 to 10^6 scotopic trolands) required to elicit a criterion ($5 \mu\text{V}$) ERG response. As shown in Fig. 4, methanol intoxication produced a progressive time- and formate concentration-dependent increase in the intensity of light required to elicit the criterion achromatic light response. In untreated-control animals, the achromatic light response was obtained at -2.9 ± 0.1 LRRI. Twenty-four hours after the initial dose of methanol, retinal responsiveness was decreased by 0.6 log units with an achromatic light response recorded at -2.3 ± 0.2 LRRI. In rats intoxicated for 48 h, retinal responsiveness was decreased by 0.9 log units with an achromatic light response recorded at -2.0 ± 0.2 LRRI, and after 72 h of intoxication, retinal responsiveness was decreased by 1.7 log units with an achromatic light response recorded at -1.2 ± 0.4 LRRI. Although, outer ret-

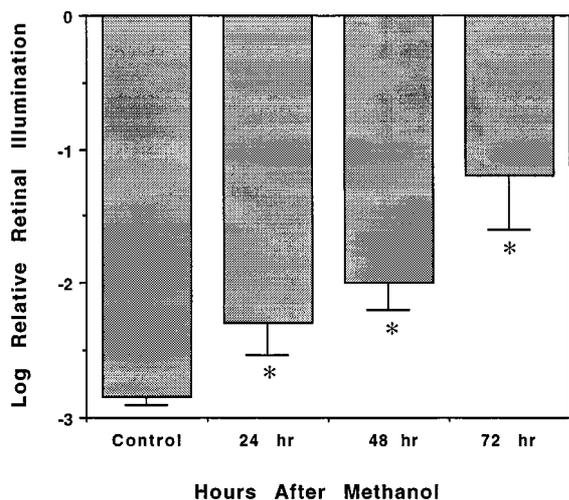


Fig. 4. Achromatic light response in methanol-intoxicated and untreated-control rats. The intensity of an achromatic light stimulus (15 Hz) required to elicit a criterion $5 \mu\text{V}$ ERG response was measured. Achromatic light responses were recorded over a 3-log unit range of light intensity. For these studies, the unattenuated stimulus (log relative retinal illumination, 0) had an irradiance of 8 mW distributed over the 70° patch of illuminated retina. By equating the effectiveness of this light to the 510-nm stimulus, we estimate that the unattenuated light produced the equivalent of 10^6 scotopic trolands in the rat eye. ERG recordings were obtained at 24, 48, and 72 h after the initial dose of methanol in the same animals in which 15Hz/510 nm and cone-mediated responses were recorded. Data are expressed as the mean values \pm S.E.M. from four to eight rats in each treatment group. Significant differences from values obtained in untreated-control rats are denoted with asterisks (Student's *t* test; $P < .05$).

inal function was profoundly attenuated in rats intoxicated for 72 h, it was not abolished at the light intensity used in these experiments. Achromatic light responses measured in N_2O -control (achromatic light response, -2.7 ± 0.1 LRR) or methanol-control animals (achromatic light response, -2.6 ± 0.2 LRR) did not differ from those measured in untreated-control rats.

Correlation Between Formic Acid Accumulation and ERG Alterations

Attenuation of both 15 Hz/510 nm and UV-cone-mediated ERG responses and increases in the intensity of an achromatic light stimulus required to elicit an ERG response occurred coincident with the linear increase in blood formate concentrations in methanol-intoxicated rats, indicative of a causal relationship between formic acid accumulation and retinal dysfunction. The data in Fig. 5 show the relationship between the maximal amplitude of 15 Hz/510 nm (Fig. 5A) and UV-cone-mediated (Fig. 5B) ERG responses and the corresponding concentration of formate in the blood of control and methanol-intoxicated rats. A highly significant negative correlation was demonstrated between blood formate concentration and each of these parameters of retinal function. These findings are indicative of a concentration-dependent disruption of rod- and cone-mediated retinal responses by formate under conditions in which blood and tissue levels of formate are steadily increasing. It is also apparent from these data that 15 Hz/510 nm ERG responses are more sensitive than UV-cone-mediated retinal responses to increases in blood formate concentration. Concentrations of formate predicted from these curves to produce a 50% attenuation of ERG response were ~ 4 mM for 15 Hz/510 nm

retinal responses and 6 mM for UV-cone-mediated responses. Formate concentrations predicted to extinguish the 15 Hz/510 nm and UV-cone-mediated responses were 8 and 12 mM, respectively. A linear relationship between blood formate concentration and the intensity of achromatic light required to elicit a $5 \mu\text{V}$ ERG response was also apparent in methanol-intoxicated rats. Figure 5C presents the relationship between blood formate concentrations and the achromatic light response. In methanol-intoxicated rats, there was a formate concentration-dependent increase in the intensity of achromatic light required to elicit the achromatic light response. The intensity of achromatic light required to evoke the criterion ERG response was elevated 0.6, 0.9, and 1.7 log units as formate concentration increased. Formate concentrations predicted from this curve to increase the response threshold by 1 and 2 log units were 5 and 10 mM, respectively. A formate concentration of 14 mM would be predicted to extinguish the response.

Effects of Formate Exposure on Retinal Histology and Photoreceptor Ultrastructure

The effects of increasing formate concentrations after methanol intoxication on retinal histology and ultrastructure were assessed by light and electron microscopy at 24, 48, and 72 h after the initial dose of methanol. The most pronounced alterations observed in the neural retina of methanol-intoxicated rats at the light microscopic level was the development of retinal edema, swelling of photoreceptor inner segments, and morphologic changes in photoreceptor nuclei. Figure 6 illustrates outer retinal morphology in representative control (Fig. 6A) and methanol-intoxicated rats at 24 h (Fig. 6B), 48 h (Fig. 6C), and 72 h (Fig. 6D) after the initial dose of methanol. Retinal histology in 24-h methanol-intoxicated rats was indistinguishable from that observed in control animals. At 48 h, the retinas of methanol-intoxicated animals appeared diffusely edematous by light microscopy, as evidenced by the spacing between the inner segments and the spacing of the nuclei in the outer nuclear layer. Evidence of swelling in the photoreceptor inner segments was also apparent at 48 h. At 72 h, photoreceptor inner segments were profoundly swollen and enlarged. Changes in the appearance of the photoreceptor nuclei were also apparent at this time. Nuclei appeared somewhat enlarged with irregularly stained chromatin. The chromatin staining pattern in photoreceptor nuclei in 72-h intoxicated animals ranged from tightly compact to dispersed and fragmented. No similar morphologic alterations were apparent in photoreceptor nuclei from control animals. In addition, no histopathologic changes were apparent at the light microscopic level in the N_2O or methanol-control groups.

Ultrastructural Alterations. The retinas of control and methanol-intoxicated rats were also examined by electron microscopy. Ultrastructural investigations focused upon the inner segments of the photoreceptors because this was the area of the outer retina that showed the most profound changes at the light microscopic level. The most obvious ultrastructural change observed in the outer retina of methanol-intoxicated rats was the swelling and disruption of photoreceptor mitochondria, which was most evident in rats intoxicated for 72 h. Figure 7 illustrates the rod inner segment region in representative control (Fig. 7A) and methanol-intoxicated rats 24 h (Fig. 7B), 48 h (Fig. 7C), and 72 h

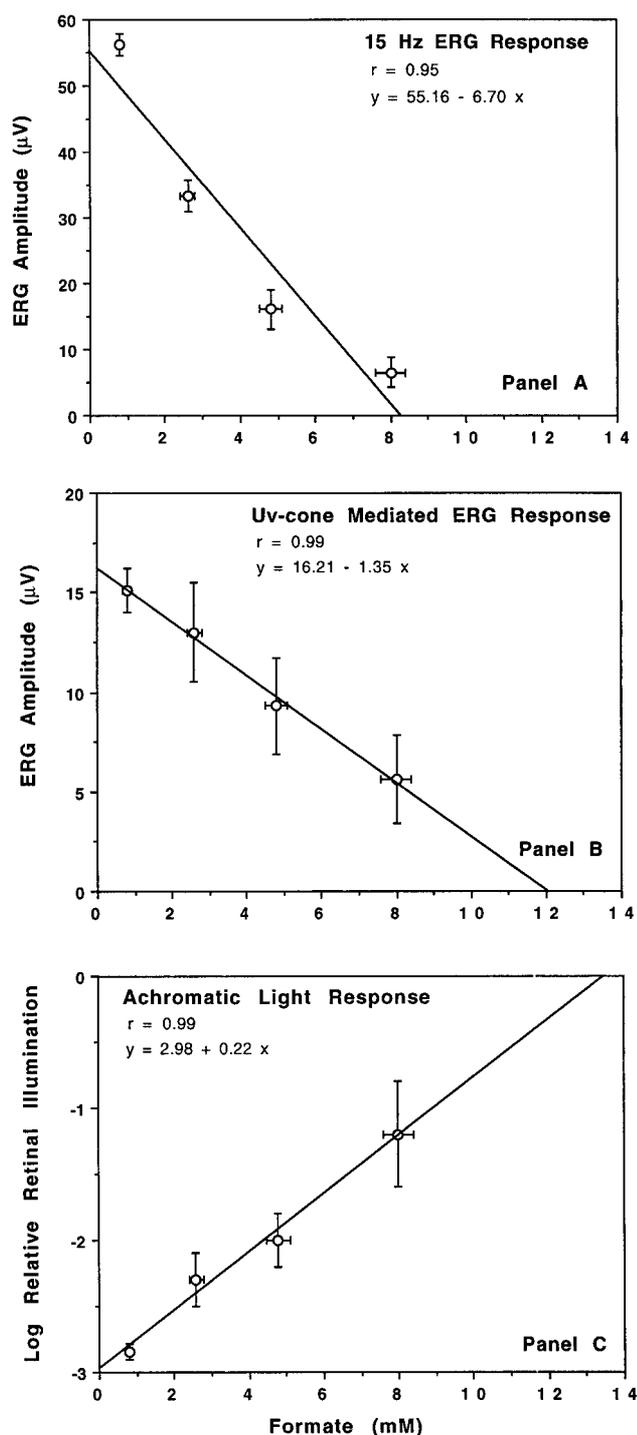


Fig. 5. Correlation of ERG response amplitude and achromatic light response threshold with blood formate concentrations in methanol-intoxicated rats. The data illustrate the relationship between blood formate concentrations and the maximal amplitude of the 15 Hz/510 nm ERG response (A), the UV-cone-mediated ERG response (B), and the intensity of an achromatic light stimulus (15 Hz) required to elicit a threshold ERG response (C) in methanol-intoxicated rats. For A and B, each point represents the value for the maximal ERG amplitude (mean \pm S.E.M.; μ V) and corresponding blood formate concentrations (mean \pm S.E.M.; mM) measured in four to six methanol-intoxicated rats at 0, 24, 48, and 72 h after the initial dose of methanol. For C, each point represents the log relative retinal illumination (mean \pm S.E.M.) and corresponding blood formate concentrations (mean \pm S.E.M.; mM) measured in four to six methanol-intoxicated rats at 0, 24, 48, and 72 h after the initial dose of methanol. In each case, the correlation coefficient is indicated and is highly significant ($P < .01$).

(Fig. 7D) after the initial dose of methanol. Photoreceptor mitochondria from untreated-control, N_2O -control, or methanol-control rats showed normal morphology with well-defined cristae. No alterations in photoreceptor ultrastructure or mitochondrial morphology were apparent in rats intoxicated for 24 h. In rats intoxicated for 48 h, vacuoles formed by dilatation of the intercellular spaces were apparent in the inner segments of the photoreceptors. Also at 48 h, mitochondria of the inner segments, which were elongate with defined cristae in the control and 24-h intoxicated animals, appeared more rounded with expanded cristae. In rats intoxicated for 72 h, numerous photoreceptor mitochondria were profoundly swollen, with severely disrupted cristae, or were swollen with no apparent evidence of cristae.

Discussion

Formic acid is the toxic metabolite responsible for the retinal and optic nerve toxicity produced in methanol intoxication. Previous investigations in our laboratory have documented formate-induced retinal dysfunction and histopathology in a rodent model of methanol intoxication. The present study was undertaken to investigate the time and concentration dependence of formate-induced retinal toxicity, the nature of the functional deficits produced, and the differential sensitivity of retinal cell types to the cytotoxic actions of formic acid. Several important findings are reported. Results indicate that retinal dysfunction occurs early in the course of intoxication at formate concentrations significantly lower than those associated previously with retinal or optic nerve dysfunction. Rod- and cone-mediated ERG responses were attenuated in a formate concentration- and time-dependent manner, and both retinal sensitivity and maximal responsiveness to light were diminished. Both 15 Hz/510 nm and UV-cone-mediated ERG responses were undetectable by 72 h; however, if light intensity was increased, a retinal ERG response could be recorded, indicating that photoreceptor function was profoundly attenuated but not abolished under these intoxication conditions. Finally, functional changes preceded structural alterations. Histopathological changes were most pronounced in the outer retina, with evidence of inner segment swelling, photoreceptor mitochondrial disruption, and the appearance of fragmented photoreceptor nuclei in the outer nuclear layer. The nature of both the functional and structural alterations observed are consistent with formate-induced inhibition of mitochondrial energy production, resulting in photoreceptor dysfunction and pathology.

In the present studies, retinal dysfunction was detected early in the course of intoxication at formate concentrations significantly lower than those associated previously with retinal or optic nerve dysfunction. Reproducible decrements in 15 Hz/510 nm ERG responses were apparent as early as 24 h after the initial dose of methanol at a mean blood formate concentration of 2.6 ± 0.2 mM. Formate-induced reductions in the flicker-evoked ERG also occurred at formate concentrations lower than those required to diminish the flash-evoked cortical potential in methanol-intoxicated rats (Eells, 1991) and lower than those associated with retinal and optic disc edema, pupillary dilatation, or histopathologic alterations in the retina or optic nerves in humans or nonhuman primates (Hayreh et al., 1980; Sharpe et al., 1982; Eells et al., 1991). We attribute this improvement in sensitivity to the

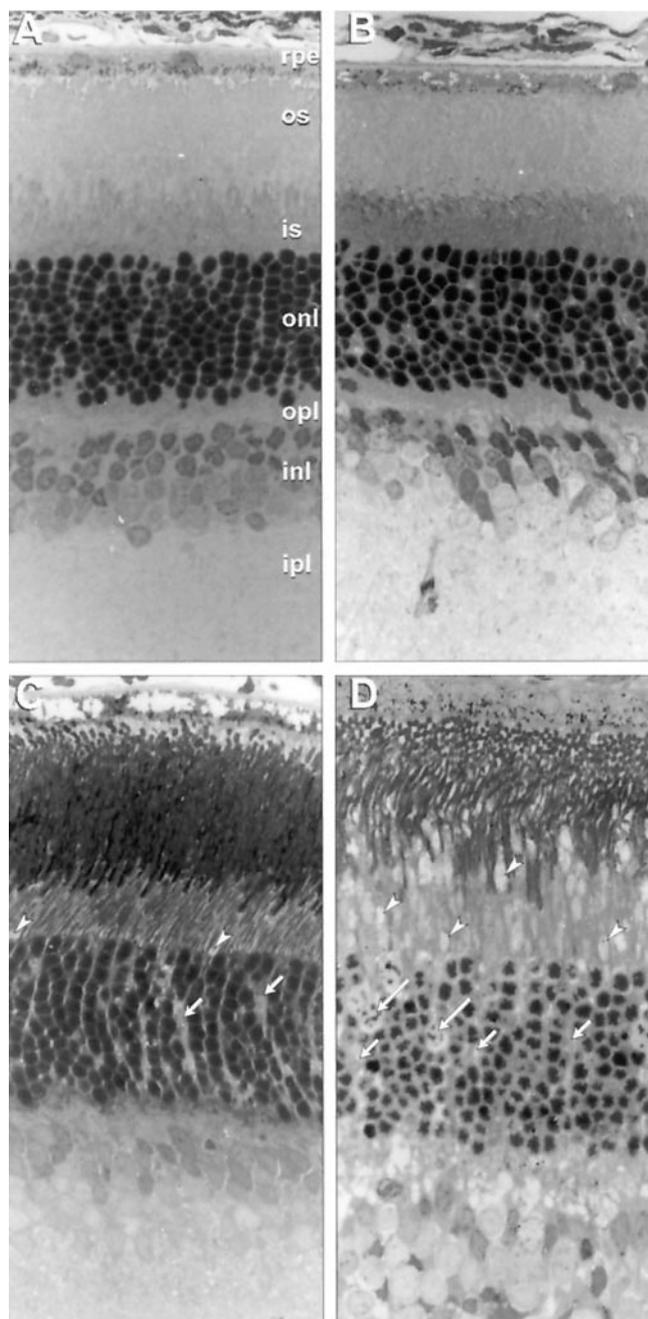


Fig. 6. Effect of methanol intoxication on retinal histology. Light micrographs of retinal tissue prepared from untreated-control rat (A) and methanol-intoxicated rats at 24 h (B), 48 h (C), and 72 h (D) after the initial dose of methanol. Sections were taken from the posterior pole of the retina within two disc diameters of the optic nerve in any direction. A: rpe, retinal pigment epithelium; os, photoreceptor outer segments; is, photoreceptor inner segments; onl, outer nuclear layer; opl, outer plexiform layer; inl, inner nuclear layer; ipl, inner plexiform layer. The short arrows indicate areas of retinal edema, as evidenced by increased spacing between the nuclei in the outer nuclear layer. The arrowheads indicate enlargement and swelling of the photoreceptor inner segments, and the long arrows indicate the fragmented appearance of photoreceptor nuclei. (Toluidine blue, 450 \times)

technique used to assess retinal function in the present experiments. These experiments measured flicker-mediated ERG responses over an extended range of light intensity, thus enabling the assessment of both retinal sensitivity to light and maximal retinal responsiveness to light. These

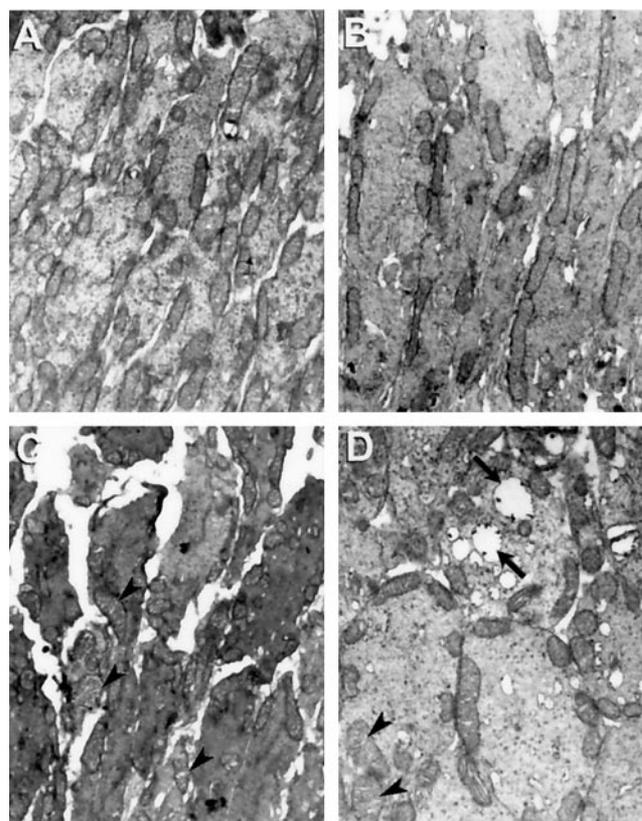


Fig. 7. Effect of methanol intoxication on ultrastructural histology. Electron micrographs of the rod inner segment region of retinal tissue prepared from untreated-control rat (A), and methanol-intoxicated rats at 24 h (B), 48 h (C), and 72 h (D) after the initial dose of methanol. The arrows and arrowheads indicate abnormal mitochondrial morphology in photoreceptor inner segments. The arrowheads indicate swollen mitochondria with expanded cristae, and the arrows indicate completely disrupted mitochondria with no evidence of cristae. (5000 \times)

findings have important implications in terms of both acute and chronic exposure to methanol, because they provide evidence of retinal dysfunction at blood formate concentrations that are presently presumed to be nontoxic (Kavet and Naus, 1990; Eells, 1992).

The results of these ERG studies strongly suggest that photoreceptor function is disrupted by formate in methanol intoxication. The observed loss of retinal sensitivity and response amplitude is consistent with a disruption of the activation of the phototransduction cascade coupled with an attenuation in saturated photoresponse. Similar functional alterations are produced by treatments known to disrupt photoreceptor metabolism (Torre, 1982; Fox and Katz, 1992). Furthermore, disruption of photoreceptor metabolism is consistent with the mechanism by which formate is postulated to produce retinal and optic nerve toxicity. Formate has been hypothesized to produce retinal and optic nerve toxicity by disrupting mitochondrial energy production as a consequence of its inhibitory action on cytochrome oxidase, the terminal electron acceptor in the electron transport chain. Photoreceptors are the retinal cell type most dependent upon oxidative mitochondrial metabolism for maintenance of cellular function. Studies showing reductions in retinal cytochrome oxidase activity and retinal ATP concentrations (Eells et al., 1995; Garner et al., 1995) in methanol-intoxicated rats strongly support the hypothesis that formate acts

as a mitochondrial toxin in the retina. Importantly, attenuation of photoreceptor-mediated responses occurred concomitantly with the accumulation of formate and showed a strong correlation with formate blood concentrations.

In the present experiments, we observed a loss of both retinal sensitivity to light and response amplitude. We postulate that formate-induced inhibition of photoreceptor energy metabolism could account for both changes. A decrease or depletion of photoreceptor GTP, ATP, or both has been shown to affect the activation steps in the phototransduction cascade, by slowing the closing of the light-sensitive cGMP-gated cation channels in the photoreceptor outer segments (Koskelainen et al., 1994). Such a change in activation would result in a decrease in photoreceptor sensitivity to light, consistent with the increase in ERG threshold produced in the present studies (Findl et al., 1995). Attenuation of the response amplitude could also be caused by a formate-induced suppression of the dark current (Fox and Katz, 1992). The dark current is driven by Na^+/K^+ pumps in the inner segment that require ATP to pump Na^+ out and K^+ in. A decrease or depletion of ATP would inhibit the function of the Na^+/K^+ ATPase in the inner segment, leading to a disruption of current flux and dark current attenuation (Torre, 1982). Elevation of inner segment Na^+ concentration would also be anticipated to decrease or slow $\text{Na}^+/\text{Ca}^{2+}$ exchange, resulting in increased intracellular Ca^{2+} , which would down-regulate cGMP, producing a further decrease in the dark current (Capovilla et al., 1983; Tessier-Lavigne et al., 1985).

In the rat, rods and M-cones have very similar spectral sensitivities; consequently, there is no way to be certain that we have separated the response of rods from M-cones in evaluating the 15 Hz ERG response at 510 nm. However, UV-cones have a spectral sensitivity that is unique from rods and M-cones; therefore, ERGs recorded under UV-cone-isolating conditions reflect a pure cone response. Formate disrupted both 15 Hz/510 nm and UV-cone-mediated retinal responses; however, 15 Hz/510 nm retinal responses were affected earlier in the course of intoxication and at lower formate concentrations than cone-mediated responses. These observations are consistent with reports that cones are less sensitive than rods to metabolic insults, which disrupt energy metabolism (Graymore and Tansley, 1959; Fox and Katz, 1992). They also agree with the relative lack of damage to the mitochondria of cones relative to rods, which we have reported in human methanol intoxication (Eells et al., 1991). Known metabolic differences in rods and cones may explain the differential sensitivity observed in 15 Hz/510 nm and cone-mediated responses. The $\text{Na}^+/\text{Ca}^{2+}$ exchanger in rods has been reported to be five times slower than cones (Yau and Baylor, 1989). Consequently, the elevation of Ca^{2+} in rod photoreceptors would be likely to be far more pronounced than that in cone photoreceptors, resulting in a greater suppression of the dark current and photoresponse. In addition, cones contain more mitochondria than rods, and thus may have a greater metabolic reserve to enable them to continue to function for a longer period of time in the presence of a metabolic toxin (Kageyama and Wong-Riley, 1984).

The morphologic changes observed in the present study are also consistent with formate-induced inhibition of photoreceptor energy metabolism. The most striking ultrastructural alteration observed in the retinas of methanol-intoxicated rats was mitochondrial swelling and disruption in the inner

segments of the photoreceptor cells. Photoreceptors are highly metabolically active, and a disruption of ion pumping and ionic homeostasis secondary to inhibition of cytochrome oxidase activity would be anticipated to produce such morphologic alterations (Tessier-Lavigne, 1991; Ames et al., 1992). Similar morphologic alterations have been reported in the retinas of patients with mitochondrial diseases that inhibit electron transport (Runge et al., 1986; McKelvie et al., 1991) and in certain forms of light-induced retinal degeneration in which inactivation of cytochrome oxidase is postulated to play a role in the pathology (Rapp et al., 1990; Pautler et al., 1990).

The clinical features of methanol intoxication are remarkably similar to those of Leber's hereditary optic neuropathy, nutritional amblyopia, and the recent Cuban epidemic of optic neuropathy (Sadun et al., 1994; Rizzo, 1995). We hypothesize that a common pathophysiological mechanism involving impaired mitochondrial function contributes to the retinal and optic nerve dysfunction characteristic of each disease or syndrome. In Leber's hereditary optic neuropathy, the defect has been associated with mitochondrial mutations affecting subunits of complex I of the mitochondrial respiratory chain, culminating in acute bilateral central vision loss. In both nutritional amblyopia and in the Cuban epidemic of optic neuropathy, the combination of folate and vitamin B₁₂ deficiencies may result in increased endogenous formic acid concentrations due to the disruption of one carbon metabolism (Sadun et al., 1994; Rizzo, 1995). In support of this interpretation, we have reported recently both folate deficiency and formic acidemia in Cuban epidemic optic neuropathy patients manifesting retinal dysfunction and optic neuropathy (Eells et al., 1996a). Therefore, understanding the mechanism of formate-induced mitochondrial dysfunction may not only improve our understanding of the pathogenesis of methanol intoxication but may also provide valuable insight into the pathogenesis of other acquired and genetic retinal and optic nerve diseases.

Acknowledgments

The excellent technical assistance of Anna Fekete is appreciated.

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