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Experiments focused on the effects of cyanide on energy metabolism, brain edema and histology. Wistar rats were infused iv with NaCN until the animal lost its righting reflex; when righting returned, NaCN infusion was started again. The local cerebral glucose use (LCGU) was measured by the [^{14}C]-2-deoxyglucose (2-DG) procedure. A shift from aerobic to anaerobic metabolism and the oxidation-reduction state of the brain extracellular fluid (ECF) were determined by intracerebral microdialysis. Immediately after iv infusion of NaCN, there was a robust increase in glucose use. Also, there was a dramatic increase, up to 5-fold, in lactate in the ECF indicating an increase in anaerobic metabolism. Sixty min after NaCN, LCGU was markedly reduced in all 46 brain regions measured, except for choroid plexus. Thus, the expected increase in glucose use as a result of anaerobic metabolism appeared to be brief. The time course profile of the [^{14}C]-2-DG in the ECF shows that glucose transport across the blood brain barrier was not inhibited by NaCN. In fact, there was a higher concentration of the [^{14}C]-2-DG in the ECF at a time when the LCGU was extremely low, indicating that both

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19. ABSTRACT

aerobic and anaerobic brain metabolism were depressed. The decrease in LCGU may be accompanied by a decrease in energy utilization that could provide a degree of "protection" from excessive neurological damage. Indeed, there does not seem to be extensive neuropathology from the NaCN exposure used. There was only transient edema and minimal damage (except thalamus) after cyanide assessed by LCGU and H&E stain. Inhibition of aerobic metabolism by cyanide could promote the formation of reactive oxygen species. Thus, we will determine how cyanide affects the formation of ascorbate and hydrogen peroxide in the ECF.

FOREWORD

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INTRODUCTION

Cyanide is a rapidly acting metabolic poison that among other things interferes with the oxidative generation of free energy in cells. Although the exact chemical details are still unknown, the undissociated form (HCN) appears to block electron transfer in the cytochrome a-a_3 complex (Schubert and Brill, 1968; Isom and Way, 1984; Piantadosi *et al.*, 1983). Acute cyanide intoxication produces incoordination of movements, convulsions, tremors, changes in EEG, coma, cardiac arrhythmias and respiratory arrest (Burrows *et al.*, 1982; Persson *et al.*, 1985; Brierley *et al.*, 1976, 1977), all of which are signs that the central nervous system (CNS) is an early target of HCN toxicity. The brain is particularly vulnerable to cyanide because of its limited anaerobic metabolic capacity and high energy dependence (Johnson *et al.*, 1986; Way, 1984). In fact, lethality from cyanide poisoning results from failure of vital functions of the CNS. Cyanide produces a histotoxic energy failure that is associated with cellular swelling, elevated brain calcium levels and increased lipid peroxidation (Johnson *et al.*, 1986; 1987). CNS areas that appear to be vulnerable to cyanide-induced damage include: layer IV cortex, hippocampus CA_1, caudate, putamen, globus pallidus, corpus callosum, optic chiasm, and substantia nigra (Hicks, 1950; Levine, 1967; Hirano *et al.*, 1967; Funata *et al.*, 1984; Bass, 1968). Thus, survivors of cyanide intoxication would be prone to have extensive neuronal damage and consequent motor and/or behavior deficits.

The problem under investigation in this contract is the neurochemical mechanism(s) that underlie the neuropathological consequences of sublethal doses of cyanide. The experimental model was adult male laboratory rats exposed to sodium cyanide by controlled intravenous infusion. The approach is measuring changes in brain regional metabolism with the 2-deoxyglucose procedure and monitoring changes in chemical composition of the extracellular

compartment with the intracerebral microdialysis procedure and to relate these to neurotoxicological changes that occur. Although there are reports on changes in tissue chemical composition after cyanide exposure, this proposal is unique in that the extracellular microenvironment of cells is the subject of study. Moreover, the 2-deoxyglucose method gives a complete visual map of metabolism through the brain and level of glucose use is quantitated in 46 discrete brain regions.

BODY

Experimental Methods:

Animals: Adult male Wistar rats weighing 250-300 g were obtained from SASCO Co. (Omaha, NE). Food and water were provided *ad libitum* and a 12-hour light/dark cycle was maintained. Experiments were conducted in accordance with the guidelines of the National Research Council DHEW Publication No. (NIH) 80-23 (1980).

Agent: Sodium cyanide was obtained from Fisher Scientific Co. (Fairlawn, NJ). Cyanide was used in accordance with the security assurances required. All unused sodium cyanide solutions and contaminated material was alkalinized with 0.1 N NaOH to prevent the escape of hydrogen cyanide (Robinson *et al.*, 1984).

Sodium cyanide exposure: Rats were given saline or NaCN by controlled intravenous infusion (20 μl/min; 4.5-5.0 mg/ml) into the femoral vein. The infusion was temporarily halted when the rat lost its righting reflex and resumed when righting recurred.

Brain regional metabolism mapping: The method for regional brain glucose utilization was based on that of Sokoloff *et al.* (1977). [^{14}C]2-Deoxyglucose (specific activity = 55 mCi/mmol; American Radiolabeled Chemicals Inc., St. Louis, MO) was injected i.v. (100-150 μCi/kg) as a pulse in 0.9% saline. During the first minute immediately following the pulse, six timed serial arterial blood samples (50-70 μl) were collected in heparinized hematocrit tubes. Blood samples were taken every 5 minutes for plasma glucose determinations and ^{14}C scintillation counting. At the end of the experiment, the rat was decapitated and the brain quickly removed, frozen in freon 12 at -70°C and bagged in plastic airtight bags for storage at -70°C.

Five microliters of each plasma sample was pipetted into 4 ml of scintillation cocktail (Research Products International Corp., Econo-Safe) and counted in a Hewlett-Packard Tri-Carb

Scintillation Counter. Ten microliters of plasma was used to determine plasma glucose levels with a Yellow Springs Instrument Model 23A glucose analyzer (Yellow Springs, OH).

Coronal sections (20 μm) of brain were cut at -20°C and immediately dried on a 55-60°C slide warmer. Adjacent sections were used for autoradiograms and hematoxylin and eosin (H&E) stains. These sections, along with [^{14}C]methyl methacrylate standards, were exposed to Kodak Min-R X-ray film for 21 days. The optical density of a given brain structure was determined by video-computer assisted analysis. For each subject, the average of several optical density readings per brain area, the plasma glucose level and plasma ^{14}C concentration were used to calculate local cerebral glucose utilization (LCGU) according to the equation developed by Sokoloff *et al.* (1977). The equation and constants used are given in Figure 1.

Microdialysis fiber assembly: The microdialysis fibers can be purchased from Bioanalytical Systems Inc. (West Lafayette, IN); however, we have extensive experience preparing our own fibers and thus can modify the fibers for specific purposes. Briefly, dialysis fiber loops were prepared from Dow 50 cellulose tubing (outer diameter = 250 μm; molecular weight cutoff = 5000) with a stainless steel wire (diameter = 0.0035 inch) passing through the lumen for support. The fiber is inserted and glued (with epoxy) into two 2-cm sections of stainless steel tubing (23 gauge), leaving 6 mm of exposed dialysis fiber length. Fiber assemblies were wetted inside first, with Krebs-Ringer bicarbonate (KRB; see below for composition) and folded into a loop configuration just before implantation.

$$\text{Rate of Reaction} = \frac{\text{Labeled Product Formed in Interval of Time, 0 to T}}{\left[\begin{array}{c}\text{Isotope Effect}\\ \text{Correction Factor}\end{array}\right]\left[\begin{array}{c}\text{Integrated Specific Activity}\\ \text{of Precursor}\end{array}\right]}$$

Operational Equation of [^{14}C]Deoxyglucose Method:

$$R_i = \frac{\overbrace{\overbrace{C_i^*(T)}^{\text{Total } ^{14}\text{C in Tissue at Time, T}} - \overbrace{k_1^* e^{-(k_2^*+k_3^*)T}\int_0^T C_p^* e^{(k_2^*+k_3^*)t}\,dt}^{^{14}\text{C in Precursor Remaining in Tissue at Time, T}}}^{\text{Labeled Product Formed in Interval of Time, 0 to T}}}{\underbrace{\left[\frac{\lambda \cdot V_m^* \cdot K_m}{\Phi \cdot V_m \cdot K_m^*}\right]}_{\text{Isotope Effect Correction Factor}}\underbrace{\left[\underbrace{\int_0^T \left(\frac{C_p^*}{C_p}\right)dt}_{\text{Integrated Plasma Specific Activity}} - \underbrace{e^{-(k_2^*+k_3^*)T}\int_0^T \left(\frac{C_p^*}{C_p}\right)e^{(k_2^*+k_3^*)t}\,dt}_{\text{Correction for Lag in Tissue Equilibration with Plasma}}\right]}_{\text{Integrated Precursor Specific Activity in Tissue}}}$$

where

R_i = glucose consumption per gm of tissue

$C_i^*(T)$ = total concentration of [^{14}C]DG and [^{14}C]DG-6-P (determined by quantitative autoradiography) in the tissues at time, T

C_p^* = arterial plasma [^{14}C]DG concentration

C_p = arterial plasma glucose concentration

k_1^* = rate constant for transport of [^{14}C]DG from plasma to tissue precursor pool

k_2^* = rate constant for transport of [^{14}C]DG from tissue back to plasma

k_3^* = rate constant for phosphorylation of [^{14}C]DG

λ = ratio of distribution volume of [^{14}C]DG to that of glucose in the tissue

ϕ = fraction of glucose that, once phosphorylated, is glycolytically and oxidatively metabolized

K_m^* = Michaelis-Menten kinetic constant of hexokinase for [^{14}C]DG

V_{max}^* = maximum velocity of hexokinase for [^{14}C]DG

K_m = Michaelis-Menten constant of hexokinase for glucose

V_{max} = maximum velocity of hexokinase for glucose

$$\frac{[\lambda \bullet Vm^* \bullet Km]}{[\phi \bullet Vm \bullet Km^*]} = 0.481 \pm 0.119$$

Values for constants in Albino Rat (mean values):

Gray matter: $K_1^* = 0.189 \pm 0.012$ (min^{-1})
$K_2^* = 0.245 \pm 0.04$ (min^{-1})
$K_3^* = 0.052 \pm 0.01$ (min^{-1})

White matter: $K_1^* = 0.079 \pm 0.01$ (min^{-1})
$K_2^* = 0.133 \pm 0.05$ (min^{-1})
$K_3^* = 0.02 \pm 0.02$ (min^{-1})

Figure 1. Operational equation of the [^{14}C]-2-deoxyglucose method. From Sokoloff, 1976; 1981.

Fiber implantation: Animals were anesthetized with pentobarbital (40 mg/kg, i.p.) and placed in a stereotaxic apparatus. Anesthesia was maintained with methoxyflurane. A hole was drilled in the appropriate place in the exposed skull to allow placement of the dialysis fiber. The stereotaxic coordinates from Paxinos and Watson (1982) relative to bregma were for right piriform cortex A - 1.8, l - 5.7, V - 9.0 mm. The animals were allowed to recover for 24 hours. The experiments were started 24 hours after the fiber implantation. After the experiment was completed, fiber placement was verified histologically.

Microdialysis perfusion: All experiments were initiated 1 day after fiber implantation and were done in unanesthetized freely moving rats. The dialysis fiber input was connected to an infusion pump with Tygon tubing and a tubing fluid swivel. The fiber was perfused with Krebs Ringer Bicarbonate (KRB, in mmol/L: NaCl, 122; KCl, 3; $MgSO_4$, 1.2; KH_2PO_4, 0.4; $NaHCO_3$, 25 and $CaCl_2$, 1.2) at a flow rate of 8 μl/min or less. Initially, the fiber was perfused with KRB alone for 2 hours. The first hour sample was discarded and the second hour sample was used to determine basal levels of the substances to be analyzed. Usually samples were collected every 30 minutes.

Lactate: Twenty microliters of perfusate was used to determine perfusate lactate concentrations by an HPLC method with UV (214 nm) detection. An Alltech Econosphere (C-18, 5 μm, 250 x 4.6 mm) column was used. The mobile phase consisted of 0.1 M sodium phosphate buffer adjusted to pH 2.4 at a flow rate of 1.2 ml/min.

[^{14}C]-2-DG in perfusate: In some rats a microdialysis fiber was implanted in the piriform cortex prior to initiation of the 2-DG procedure. In these studies radiolabel was measured in the perfusate samples.

Brain tissue specific gravity: Regional changes in brain density were measured gravimetrically by modification of the method of Nelson *et al.* (1971). Rats were sacrificed by

decapitation, the brains removed (within 2 minutes) and submerged in kerosene (4°C) for dissection. Small tissue samples (about 2 mm^3) were taken bilaterally from the frontal cortex, parietal cortex, piriform cortex, hippocampus, caudate, corpus callosum and dorsal thalamus. Samples were placed in a linear kerosene-bromobenzene gradient column and their position in the column was recorded after 2 minutes. The linearity of the column was determined with glass standards (Nelson and Olson, 1987). The increase in tissue volume, which represents edema fluid, was then calculated from change in specific gravity according to the formula:

$$\textbf{Percent change in tissue volume as water} = \left[\frac{\text{Control Sp.Gr.} - 1}{\text{Experimental Sp.Gr.} - 1} - 1\right] \text{X } 100$$

Histology: The animals were anesthetized with 40 mg/kg in pentobarbital and perfused by intracardiac injection with 10% buffered formalin. Frozen cryostat sections (20 μm) were used to verify placement of fibers. Hematoxylin and eosin stained paraffin cut sections (5 μm) were used to identify neuropathology.

Statistics: LCGU data was analyzed with a one-way analysis of variance followed by a Dunnett's t-test. Significance was set at $p < 0.05$. Data in Figures 2-5 was analyzed by repeated analysis of variance. Data in Figures 7, 8 and 10 were analyzed with a Student's t-test.

Results and Discussion:

Brain metabolism studies: To determine the brain regions affected by sublethal doses of cyanide, male rats were given saline or NaCN by controlled intravenous infusion (20 μl/min; 4.5-5.0 mg/ml) into the femoral vein. The infusion was temporarily halted when the rat lost its righting reflex and infusion resumed when righting recurred. The infusion process lasted 1 hour or until the end of the 45 minute 2-DG labelling period, depending on which came first. The amount of NaCN received by each group was: 1 minute group = 4.40 ± 0.27; 60 minute group = 7.60 ± 0.81; 6 hour group = 9.47 ± 1.39; and 24 hour group = 6.83 ± 0.76 mg/kg. A pulse of [^{14}C]-2-deoxyglucose (2-DG) was given i.v. 1 minute, 60 minutes, 6 hours or 24 hours after the NaCN infusion was started in order to determine local cerebral glucose use (LCGU). All rats were sacrificed 45 minutes after injection of 2-DG. The results are given in Table I of the appendix. LCGU was significantly increased in the cyanide (1 minute) group (Table I, column 2) in several structures. Structures that had increases greater than 150% of control include: nucleus accumbens, ventral pallidum, lateral septum, peduncular nucleus, ventral striatum, globus pallidus, basolateral amygdala, central amygdala, hypothalamus, choroid plexus, hippocampal body-CA1, CA3, CA4, dentate gyrus (molecular layer), substantia nigra, interpeduncular nucleus, vermis (dorsal lobule). The structure with the highest percent increase in LCGU was corpus callosum (375% of control). This may account for the reported greater vulnerability to damage of white matter than gray matter. Sixty minutes after NaCN, LCGU was markedly reduced in all 46 regions measured, except for choroid plexus. Most regions were between 30 to 50% of control (Table I, column 3). Six or 24 hours after NaCN, LCGU was at control levels (Table I, columns 4 and 5). Thus, the expected increase in LCGU associated with a shift from aerobic to anaerobic metabolism after cyanide was of short duration. The marked reduction in LCGU found 1 hour after NaCN was dramatic and needs further exploration.

Table I. Effect of cyanide on local cerebral glucose use (μmol/100 g/min)

Brain Area	Control (N = 12)	Cyanide 1 minute (N = 4)	Cyanide 60 minute (N = 7)	Cyanide 6 hour (N = 6)	Cyanide 24 hour (N = 6)
Olfactory Tubercle	66 ± 4	99 ± 11*	24 ± 3*	59 ± 3	62 ± 5
Ventral Striatum	80 ± 6	117 ± 13*	33 ± 7*	75 ± 5	76 ± 3
Nucleus Accumbens	56 ± 5	92 ± 6*	23 ± 4*	51 ± 3	49 ± 3
Cingulate Gyrus	89 ± 7	115 ± 14	28 ± 5*	80 ± 6	79 ± 5
Frontal Cortex	81 ± 7	102 ± 10	30 ± 4*	76 ± 3	77 ± 5
Frontal Cortex-Dorsal	90 ± 8	102 ± 11	29 ± 4*	78 ± 7	80 ± 5
Frontal Cortex-Ventral	94 ± 10	114 ± 9	35 ± 4*	82 ± 5	84 ± 6
Corpus Callosum	23 ± 3	86 ± 6*	9 ± 3*	21 ± 3	18 ± 2
Dorsolateral Caudate	91 ± 7	126 ± 13	29 ± 6*	83 ± 7	86 ± 4
Ventral Pallidum	61 ± 6	95 ± 11*	28 ± 5*	55 ± 5	54 ± 3
Piriform Cortex	78 ± 6	108 ± 16	29 ± 6*	76 ± 5	75 ± 5
Lateral Septum	46 ± 4	88 ± 8*	22 ± 4*	40 ± 4	45 ± 3
Bed Nucleus Striatum	39 ± 3	76 ± 8*	19 ± 4*	39 ± 4	47 ± 3
Globus Pallidus	45 ± 4	90 ± 9*	15 ± 3*	44 ± 5	49 ± 4
Basolateral Amygdala	73 ± 6	116 ± 12*	27 ± 4*	65 ± 5	71 ± 5
Central Amygdala	41 ± 4	74 ± 9*	16 ± 3*	40 ± 3	40 ± 3
Anteroventral Thalamus	101 ± 7	127 ± 12	35 ± 5*	92 ± 7	103 ± 5
Ventrolateral Thalamus	92 ± 7	125 ± 12*	29 ± 3*	86 ± 7	88 ± 4
Hypothalamus	53 ± 5	82 ± 7*	23 ± 4*	48 ± 4	46 ± 3
Subthalamus	81 ± 7	116 ± 9*	36 ± 5*	80 ± 6	87 ± 4
Mediodorsal Thalamus	92 ± 7	120 ± 12	31 ± 4*	87 ± 6	94 ± 6
Laterodorsal Thalamus	93 ± 7	123 ± 14	31 ± 3*	84 ± 4	93 ± 6
Mammillary Body	101 ± 7	123 ± 12	41 ± 4*	99 ± 4	98 ± 5
Lateral Habenula	91 ± 6	116 ± 12	43 ± 6*	78 ± 4	91 ± 7
Choroid Plexus	67 ± 5	159 ± 9*	53 ± 14	47 ± 4*	67 ± 7
Parietal Cortex	85 ± 7	108 ± 13	28 ± 4*	73 ± 5	81 ± 6
Hippocampal Body-CA3	55 ± 5	95 ± 11*	28 ± 7*	50 ± 5	49 ± 6
Hippocampal Body-CA4	47 ± 4	84 ± 10*	23 ± 7*	45 ± 3	44 ± 4
Dentate Gyrus (Mol)	47 ± 5	80 ± 10*	17 ± 4*	45 ± 3	44 ± 4
Dorsolateral Geniculate	81 ± 4	107 ± 10	28 ± 3*	76 ± 7	81 ± 6
Hippocampal-CA1-Ventral	54 ± 4	96 ± 13*	24 ± 4*	50 ± 3	48 ± 2
Hippocampal-CA1-Dorsal	50 ± 3	85 ± 11*	19 ± 4*	49 ± 5	48 ± 2
Entorhinal	64 ± 5	99 ± 15	27 ± 8*	55 ± 4	55 ± 2
Anterior Prectectum-Thalamus	93 ± 7	118 ± 11	40 ± 4*	82 ± 6	88 ± 6
Substantia Nigra	56 ± 4	101 ± 10*	28 ± 5*	51 ± 2	51 ± 2
Substantia Nigra-Hi	62 ± 3	107 ± 10*	33 ± 5*	58 ± 2	61 ± 3
Substantia Nigra-Lo	51 ± 4	98 ± 10*	25 ± 4*	45 ± 2	46 ± 2
Interpeduncular Nucleus	89 ± 8	149 ± 9*	47 ± 7*	95 ± 5	81 ± 3
Medial Geniculate	99 ± 7	120 ± 11	31 ± 2*	91 ± 4	103 ± 7
Superior Colliculus	77 ± 4	101 ± 10	36 ± 4*	73 ± 4	74 ± 5
Lateral Lemniscus	92 ± 6	113 ± 12	41 ± 4*	91 ± 6	92 ± 5
Presubiculum	82 ± 5	107 ± 9*	25 ± 3*	82 ± 5	73 ± 5
Inferior Colliculus	119 ± 9	133 ± 9	58 ± 3*	116 ± 4	129 ± 3
Vermis (Dorsal Lobule)	52 ± 3	81 ± 6*	24 ± 4*	53 ± 4	49 ± 3
Vermis (Lobule 1)	108 ± 6	121 ± 9	45 ± 7*	101 ± 7	98 ± 1

Data expressed as means ± S.E.
*Significantly different from control ($p < 0.05$).

To further our understanding of changes in brain metabolism during cyanide exposure, a microdialysis fiber was implanted into the right piriform cortex. In these studies, rats were infused intravenously with either saline or cyanide as described above. In addition, the fiber was perfused with KRB at a rate of 8 μl/min. Basal perfusate levels of lactate (ca 100 μM) were determined prior to cyanide exposure (see Figures 2 and 3). Cyanide infusion and 2-DG injection were given at times indicated in Figures 2 and 3. Specifically, 150 μCi/kg of [^{14}C]-2-deoxyglucose was given either 15 (Figure 2) or 60 (Figure 3) minutes after initiation of NaCN infusion. The control group received saline infusion throughout the experiment. After the injection of 2-DG, lactate (Figures 2 and 3) and [^{14}C]-label (Figure 4) were monitored in the perfusate. Glucose (Figure 5) and [^{14}C]-label (Figure 6) were monitored in the plasma. The animals were sacrificed 45 minutes after 2-DG injection, and LCGU autoradiograms are in the process of being quantitated. After the start of NaCN infusion, perfusate lactate levels increased 4-5 fold over basal levels (Figures 2 and 3). This is further evidence of a shift from aerobic to anaerobic metabolism. The fact that perfusate lactate levels rapidly decline at a time when LCGU is low (see Table I and Figure 3; cyanide--60 minute--group) suggests that both anaerobic and aerobic brain metabolism have decreased. An alternate explanation for low LCGU after 60 minutes of cyanide exposure would be that cyanide is inhibiting the transport of 2-DG into the brain, thus falsely indicating a decrease in LCGU. This was ruled out by measuring the amount of [^{14}C]-label in the extracellular fluid. As seen in Figure 4 (cyanide -60 minute group), [^{14}C]-label is actually above control when LCGU is low. Thus, 2-DG is getting into the brain but is not being used after 60 minutes of cyanide exposure. Further, the decline in plasma [^{14}C]-label (Figure 6) was slowed after cyanide infusion and appears to be related to the marked hyperglycemia produced by cyanide (Figure 5). This was most prominent in the cyanide (15 minute) group, the group where plasma was collected during the cyanide infusion.

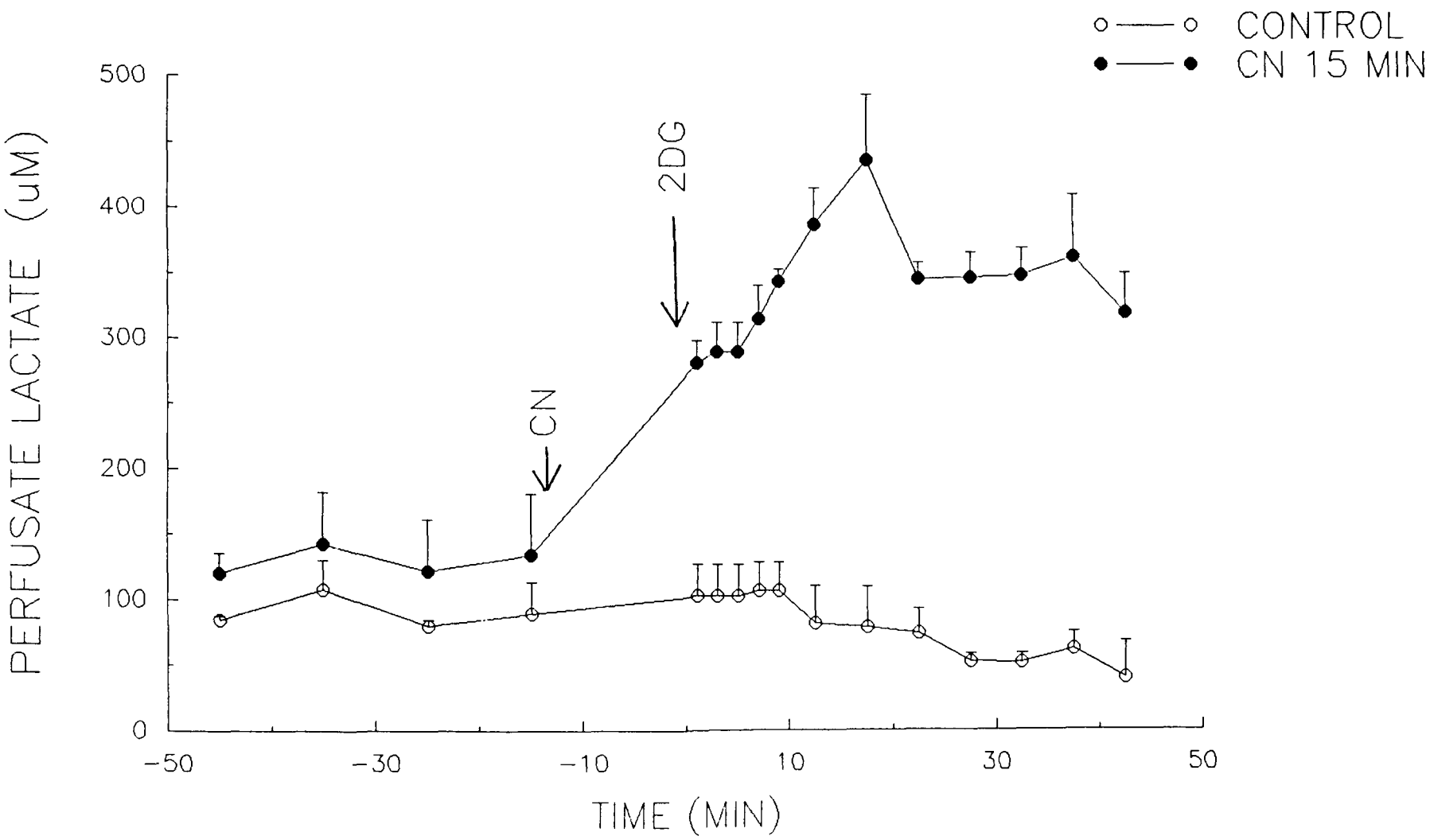

Figure 2. Concentration of lactate in microdialysis perfusate after cyanide exposure. A microdialysis fiber was implanted into the right piriform cortex. Intravenous saline infusion was initiated at -45 minutes and NaCN infusion (20 µl/min; 5 mg/ml) was initiated at time marked (CN) and given for 60 minutes as described in the text. The microdialysis fiber was perfused with KRB at 8 µl/min and lactate was measured in the perfusate. [^{14}C]-2-deoxyglucose (2-DG) was injected at time 0 which was 15 minutes after the initiation of cyanide. The rats were sacrificed 45 minutes after injection of 2-DG. Control, N = 4; cyanide (15 min), N = 3. Data was analyzed by repeated measures--ANOVA. Prior to cyanide exposure basal levels were not significantly greater than control; $p < 0.05$.

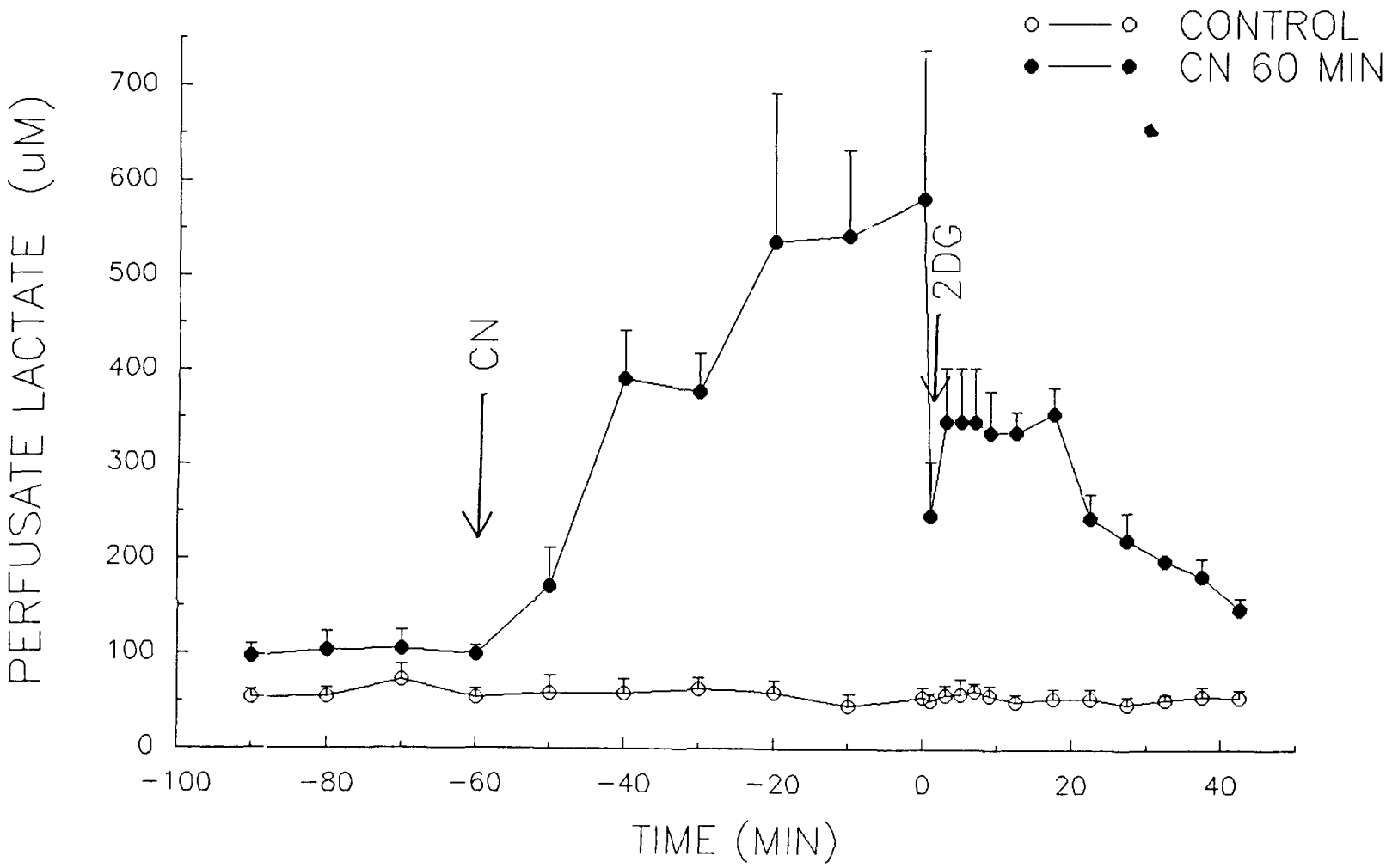

Figure 3. Concentration of lactate in microdialysis perfusate after cyanide exposure. A microdialysis fiber was implanted into the right piriform cortex. Intravenous saline infusion was initiated at -90 minutes and NaCN infusion (20 μl/min; 5 mg/ml) was initiated at time marked (CN) and given for 60 minutes as described in the text. The microdialysis fiber was perfused with KRB at 8 μl/min and lactate was measured in the perfusate. [^{14}C]-2-deoxyglucose (2-DG) was injected at time 0 which was 60 minutes after the initiation of cyanide. The rats were sacrificed 45 minutes after injection of 2-DG. Control, N = 5; cyanide (60 min), N = 4. Data was analyzed by repeated measures--ANOVA. Prior to cyanide exposure basal levels were not significantly greater than control; $p < 0.05$.

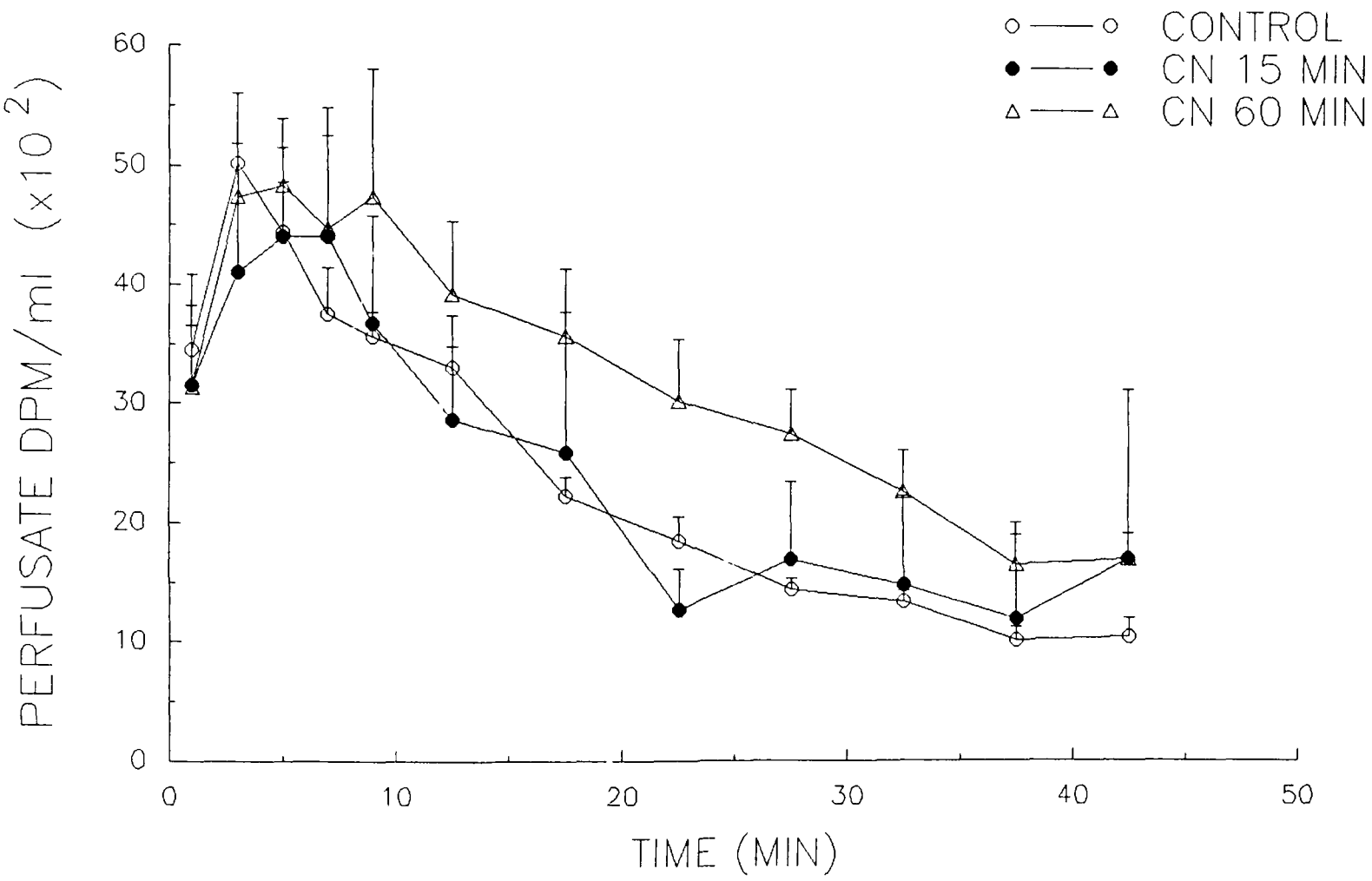

Figure 4. [^{14}C]-radioactivity in microdialysis perfusate after cyanide exposure. Rats were treated as described in Figures 2 and 3. ^{14}C-radioactivity was determined in the perfusate. Data was analyzed by repeated measures--ANOVA. Perfusate levels did not differ significantly between the three treatments ($p = 0.42$). After the initial rise, DPM values decreased over time ($p < 0.0001$). The rate of decline was not affected by treatment ($p = .90$).

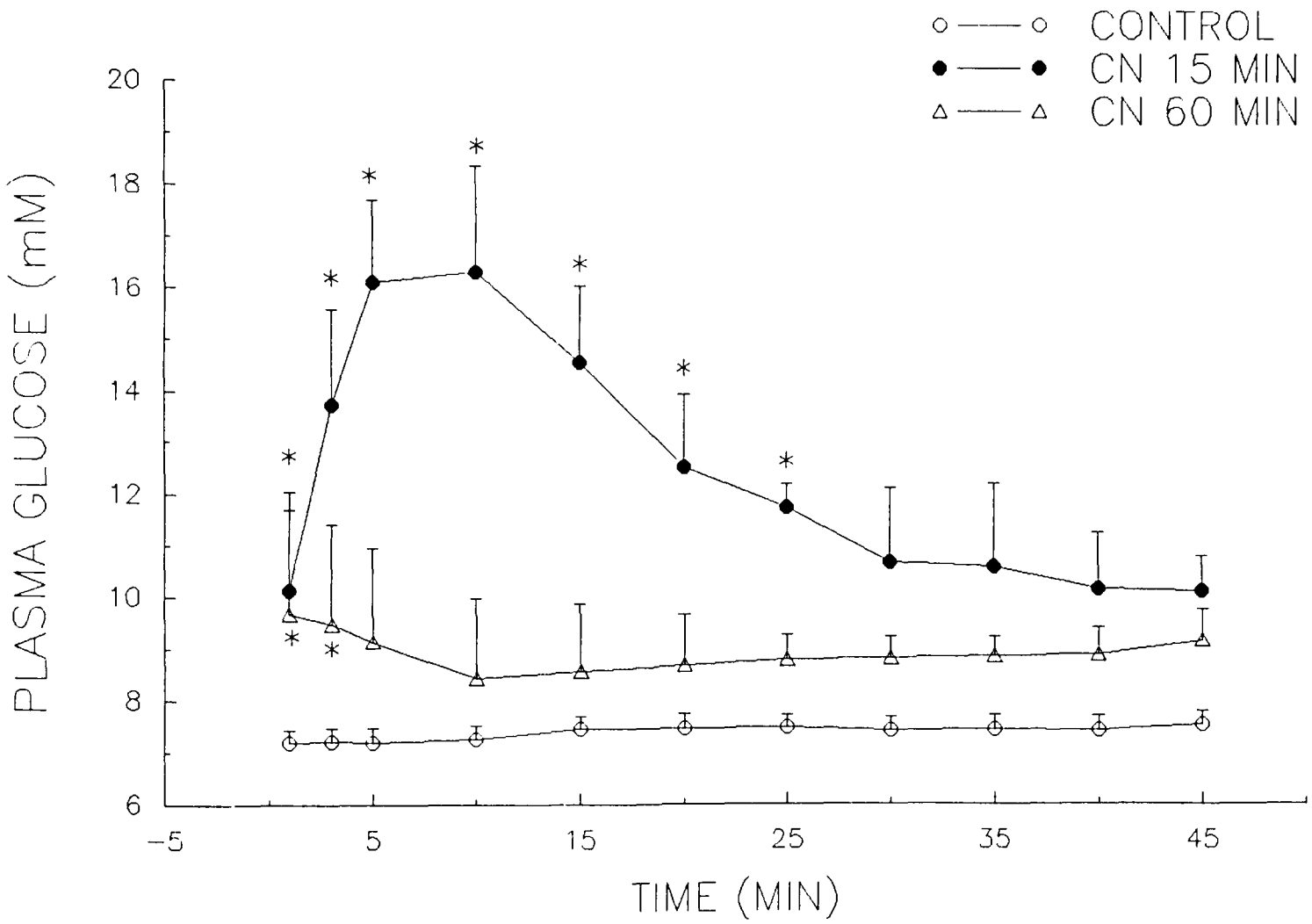

Figure 5. Concentration of glucose in the plasma after cyanide exposure. Rats were treated as described in Figures 2 and 3. Glucose levels were determined in the plasma. Data was analyzed by repeated measures--ANOVA. The treatment, time and treatment × time effects are statistically significant at the 0.05 level. When averaged over all times, the mean glucose concentration for CN 15 min is greater than CN 60 min or control treatments ($p = 0.0079$ and $p = 0.0003$, respectively). The overall mean for CN 60 min is marginally greater than the glucose concentration mean for control treatment ($p = 0.0549$). * indicates that glucose concentration is greater than control at corresponding times ($p < 0.05$).

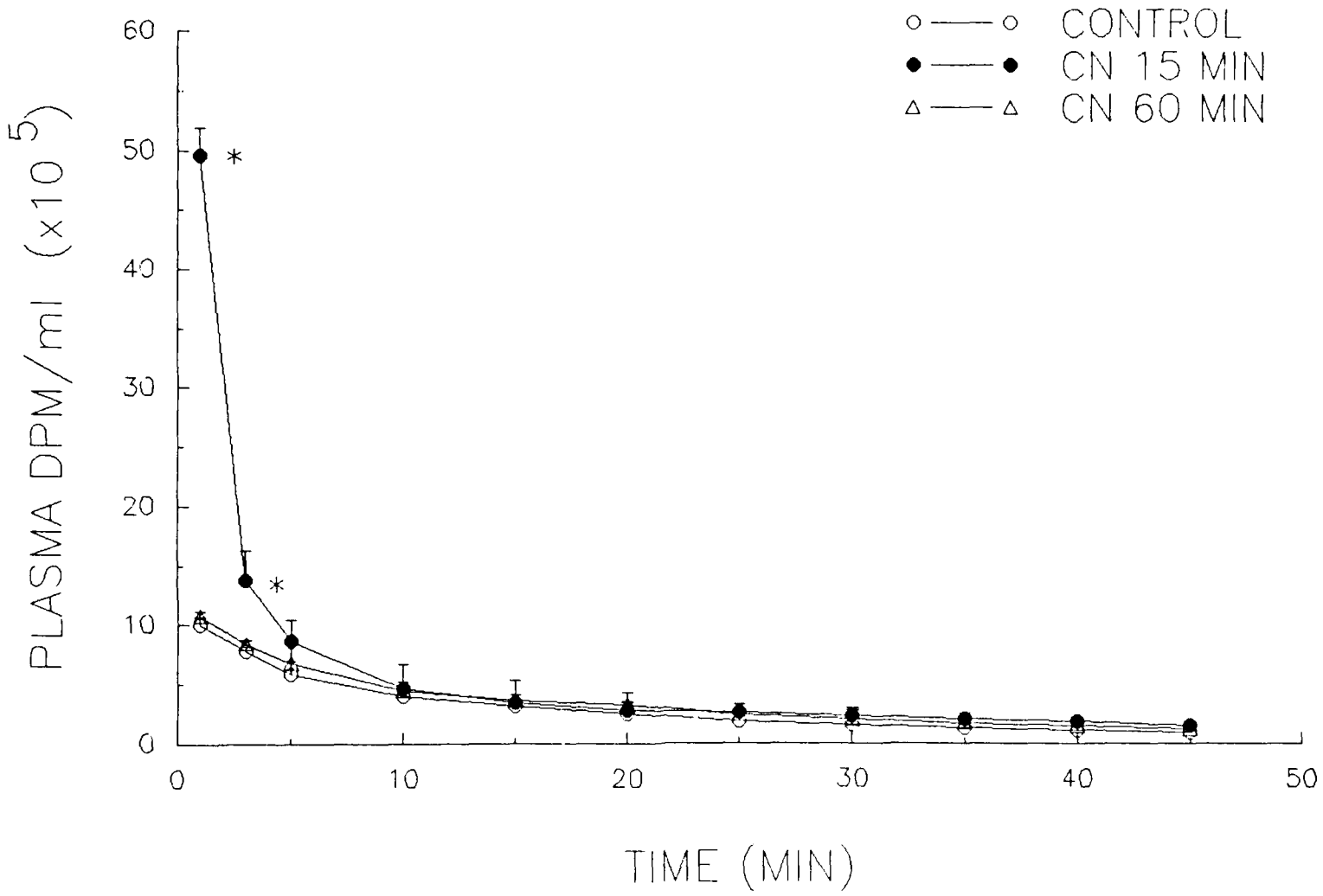

Figure 6. [^{14}C]-radioactivity in plasma after cyanide exposure. Rats were treated as described in Figures 2 and 3. ^{14}C-radioactivity was determined in the plasma. Data was analyzed by repeated measures--ANOVA. There is a statistically significant decrease in plasma DPM over time (for all treatments, $p < 0.0001$). Additionally, the treatment × time interaction is significant. Inspection of the cell-means indicates that CN 15 min is greater (*) than CN 60 min at 1 and 3 min post 2-DG and greater than control at 1, 3 and 5 min ($p < 0.05$). Differences in the three treatments at 10 min and later post 2-DG injection are not statistically significant (all p values > 0.20).

These results support the hypothesis that cyanide causes a shift in brain from aerobic to anaerobic metabolism; however, the short duration of the LCGU increase indicates that a marked depression of both aerobic and anaerobic metabolism rapidly follows NaCN exposure. We had earlier suggested that sublethal cyanide leads to increased K^+ throughout the brain within minutes after exposure and the high K^+ caused a marked depression in cerebral activity. We have determined the K^+ level in the brain at various time intervals after NaCN by intracerebral microdialysis. The extracellular K^+ does not change significantly during the time of depressed cerebral activity and thus does not account for the cerebral depression.

Brain edema studies: To determine if cyanide exposure affects energy utilization and thereby causes cellular swelling, tissue specific gravity was determined to assess brain edema as outlined below. Rats were infused with either saline or NaCN (20 μl/min; 4.5 mg/ml) into the femoral vein. The infusion was temporarily halted when the rat lost its righting reflex, and infusion was resumed when the righting reflex recurred. In the first experiment (Figure 7), rats were exposed to saline (N = 9) or NaCN (7.98 ± 1.33 mg/kg; N = 8) for 1 hour and the specific gravity of brain regions was assessed 2 hours after the initiation of the infusion. Specific gravity was slightly reduced in all brain areas and was markedly reduced in caudate and corpus callosum. Thus, these data show that there is extensive edema in the caudate and corpus callosum. The edema in corpus callosum, a primarily white matter structure, is unexpected if the major toxicity of cyanide exposure is presumed to be an interference with energy (ATP) production since the rate of energy use in white matter normally is much lower than in gray matter. The "balance" between energy reserves and rates of utilization may be involved or possibly there is a difference in the neurochemical composition of the corpus callosum.

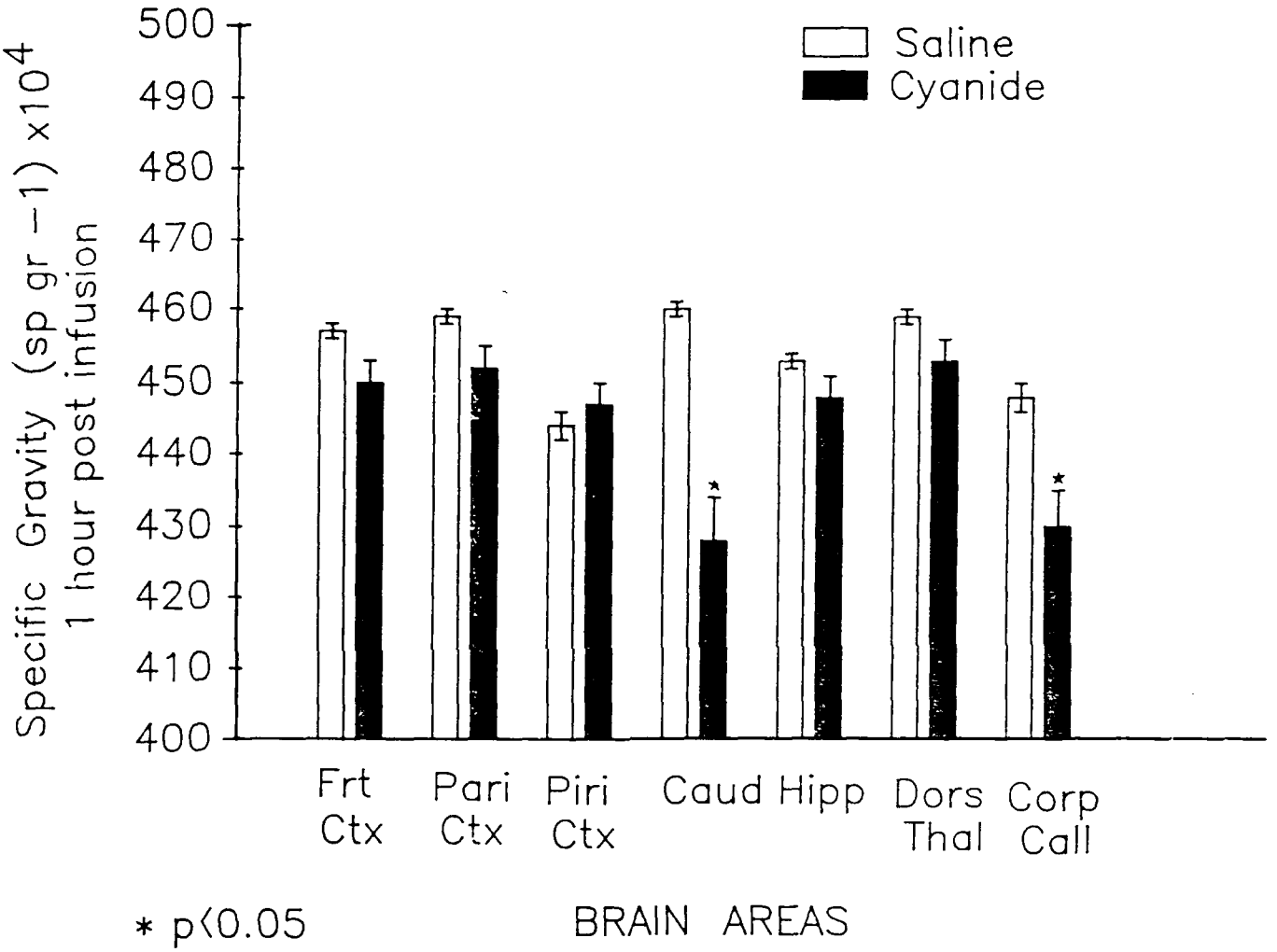

Figure 7. Specific gravity of given brain areas two hours after the initiation of a 1 hour infusion of cyanide. Rats were infused with either saline (N = 9) or NaCN (20 µl/min; 4.5 mg/ml) in the femoral vein for 1 hour as described in the text. Specific gravity was measured on different brain regions 2 hours after the initiation of NaCN infusion. * indicates that the cyanide-exposed group is significantly different from saline-exposed group (Student's t-test, $p < 0.05$).

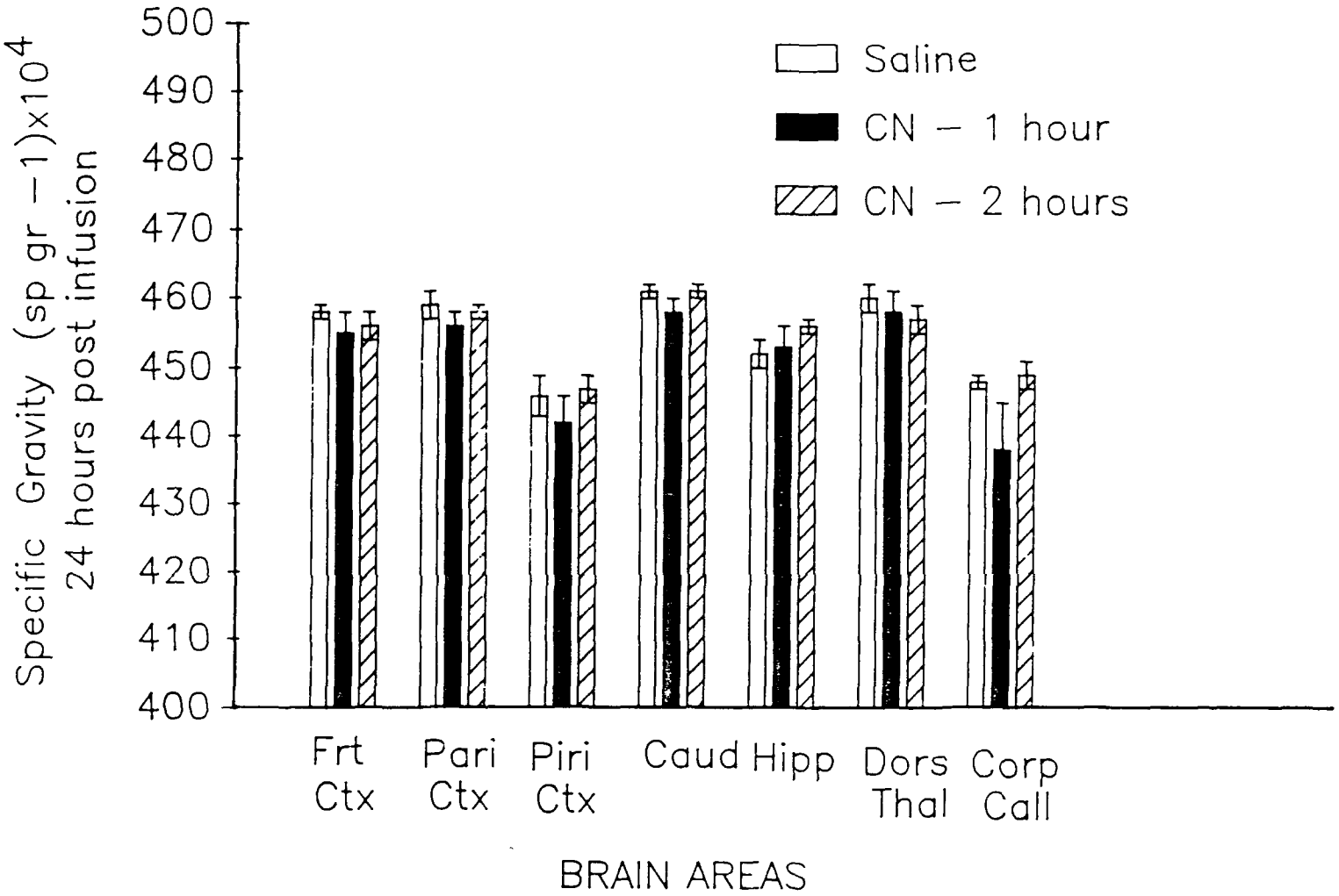

Figure 8. Specific gravity of given brain areas 24 hours after the initiation of a 1 or 2 hour infusion of cyanide. Rats were infused with saline (N = 6), NaCN (1 hour; N = 6) or NaCN (2 hours; N = 6) as described in Figure 7. Specific gravity was determined 24 hours after the initiation of NaCN infusion. The cyanide-exposed groups were not significantly different than the control group at the 0.05 level (Student's t-test).

In the second experiment (Figure 8), rats were infused with saline (N = 6), NaCN (1 hour; 5.45 ± 0.79 mg/kg; N = 6) or NaCN (2 hours; 14.27 ± 3.08 mg/kg; N = 6) and specific gravity was determined 24 hours after initiation of infusion. At this time there was no significant edema in any of the brain regions, indicating that early edema is reversible. This is in agreement with the minimal neuropathology observed in similar exposed rats.

In the third experiment rats were exposed to saline (N = 3) or NaCN (N = 15) for 2 hours per day for 4 consecutive days. Eight rats died of NaCN exposure on day 1, 1 died on day 3 and 3 died on day 4. An interesting finding was that the rats required a larger dose of cyanide on each consecutive day to lose their righting reflex. The time to loss of righting reflex for the survivors is plotted in Figure 9. On day 1, it took 6.14 ± 0.64 minutes of NaCN infusion (20 μl/min; 4.5 mg/ml); on day 2, 14.36 ± 3.50 minutes, on day 3, 27.62 ± 3.58 minutes and on day 4, 29.67 ± 6.20 min for the rats to lose their righting reflex. The 7 rats that survived the first day of cyanide exposure on subsequent days required a large dose of cyanide to cause the first loss of righting reflex. Thus, they appeared to develop a tolerance to the loss of righting reflex. It is not clear whether or not rats that survived the first day of exposure were more resistant to cyanide lethality. The experiments were not designed to determine if tolerance occurs to cyanide lethality upon daily exposure. The specific gravity of brain tissues was assessed 24 hours after the last exposure and compared to tissues taken from three control rats that had been infused with saline (see Figure 10). Two out of the three rats had a decrease in specific gravity in the caudate and corpus callosum. Thus, these two structures appear to be the most vulnerable to NaCN induced perturbations. Therefore, in the future microdialysis studies, we will focus on the caudate.

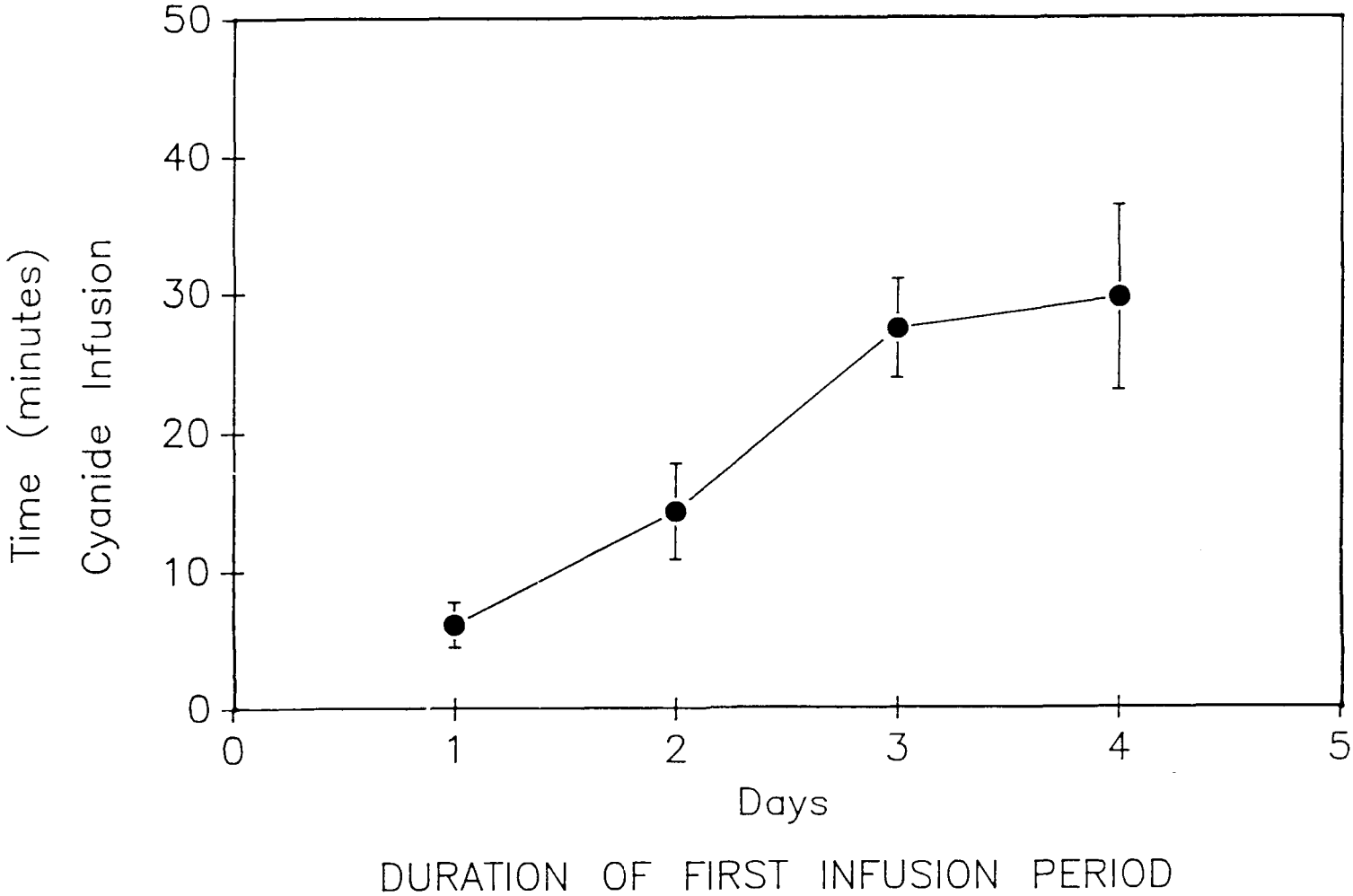

Figure 9. Duration of cyanide infusion period (minute) on consecutive days to cause the first loss of righting reflex. Rats were infused with saline (N = 3) or NaCN (N = 15) as described in Figure 7 for 2 hours/day for 4 consecutive days. Time (minutes) of cyanide infusion to cause the first loss of righting reflex is plotted for each consecutive day.

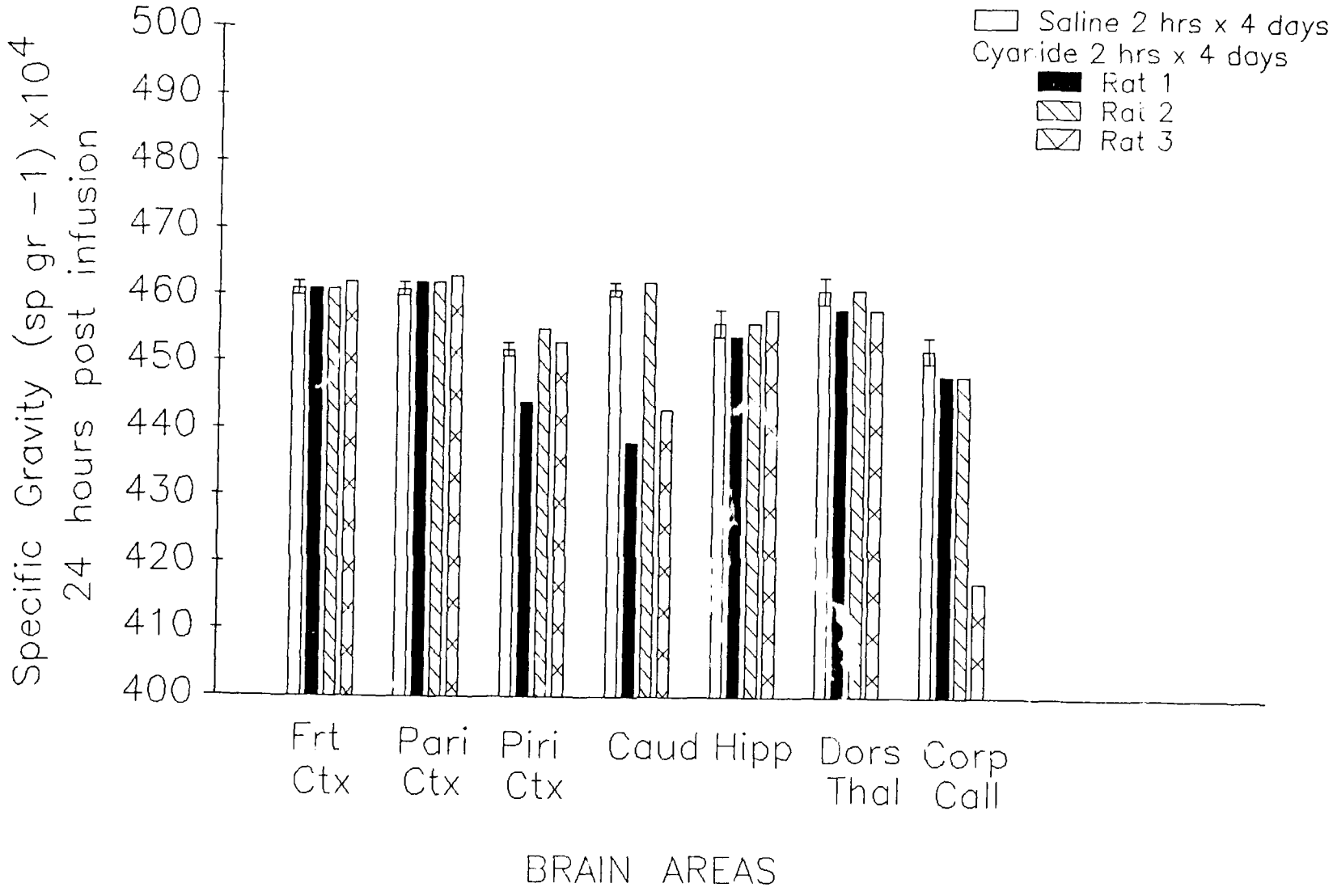

Figure 10. Specific gravity in given brain areas 24 hours after the last of 4 daily 2 hour infusion periods of cyanide. Specific gravity was determined 24 hours after the last exposure for animals that survived the infusion regimen described in Figure 9. There was a marked decrease in specific gravity in the caudate of 2 rats and in the corpus callosum in 1 rat.

Neuropathology studies: Histological studies were done on tissue from three rats exposed to cyanide for 2 hours. Control rats were infused for 2 hours with normal saline. Twenty-four hours later these rats were anesthetized with pentobarbital and the left ventricle cannulated after the thoracic cavity was opened. The brain was fixed by first clearing the vasculature with 20 ml of saline. This was followed with a mixture of paraformaldehyde (2%) and glutaraldehyde (2.5%) in 0.1 M phosphate buffer. The brain was next removed and immersion fixed in the same fixative for 3-5 days. The brain was then paraffin mounted and a central tissue block sectioned at 5 μm and stained with hematoxylin and eosin.

Examination of the tissue sections showed that the tissue structures were well maintained, at least as assessed by H & E histology. The pyramidal cell layer of the hippocampus appeared to have a few clumps of dark staining, contracted neurons, but the same changes were seen in the control rats. The white matter was compact with no evidence of demyelination or edema.

Our observation with hematoxylin and eosin stained sections did not reveal significant tissue damage 24 hours after a 2-hour intermittent cyanide exposure.

CONCLUSIONS

Studies to date support our original hypothesis that sublethal doses of cyanide cause a shift from aerobic to anaerobic metabolism. However, an even more important aspect of these studies is the marked decrease in brain metabolism after 1 hour of cyanide exposure. This marked reduction of brain activity after acute exposure of cyanide could be a double-edged sword. On the one hand, this depression of brain activity is most likely responsible for death (i.e., inhibition of central control of vital functions) whereas, on the other hand, this cyanide-induced depression of brain activity could contribute to the minimal neuropathology observed in our study as well as in others (e.g., MacMillan, 1987; Brierley, 1976, 1977) after acute cyanide exposure. This marked depression of metabolic activity that we have observed correlates well

with the cyanide induced silent EEG described by others (MacMillan, 1987; Brierley, 1976, 1977). Thus, the most important question to resolve at this point is to determine the cause of cyanide induced depression of brain activity. The microdialysis procedure is well suited towards determining if there is a chemical basis for this depression of brain activity. We suggest that sublethal doses of cyanide may produce an increase in K^+ throughout the brain within minutes after exposure and the high K^+ causes a depression in cerebral activity. Other substances such as catecholamines in the microdialysate perfusates will be analyzed.

We believe that when neuropathology does occur with cyanide exposure, it is likely to involve the role of ROS. We have made good progress in the detection of hydrogen peroxide and ascorbate in the ECF via intracerebral microdialysis. Thus, future studies will focus on the improvement of technology to detect ROS in extracellular fluid. There is good reason to believe that cyanide toxicity induces lipid peroxidation (Johnson *et al.*, 1987). Our recent success with intracerebral microdialysis technology in the detection of important oxidative (e.g., hydrogen peroxide) and reductive (e.g., ascorbate) substances in the ECF provides a new window for determining the effects of cyanide on free-radical-induced brain damage. This is of importance in discovering what situations (e.g., oxygen therapy, drugs) may amplify or protect against cyanide induced neurotoxicity.

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