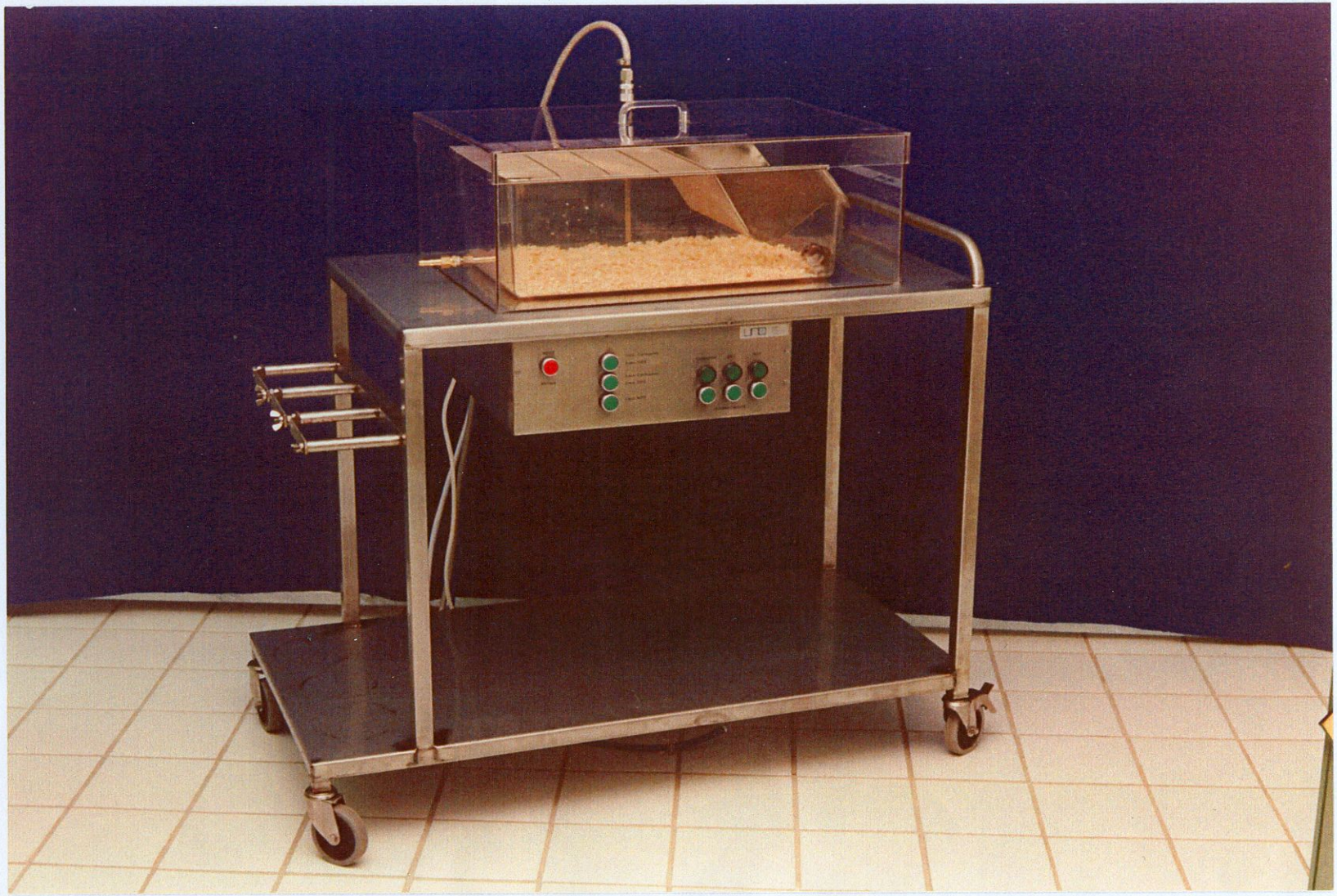
Euthanasia unit



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Carbon Dioxide-Induced Anesthesia Has No Effect on Brain Biogenic Amine Concentrations in Mice

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Ensuring a humane death for laboratory animals used in research experiments is important to the experimenter. To aid researchers in such decisions, the American Veterinary Medical Association (AVMA) convenes panels to set guidelines for selection of appropriate methods of euthanasia. The AVMA Panel on Euthanasia suggests that several criteria be considered in choosing an appropriate method of euthanasia, the principal criterion being the ability to induce loss of consciousness and death without causing pain or distress (1).

In experiments involving mice in which the integrity of the brain tissue is of concern, the animal is commonly killed by manual cervical dislocation, followed by decapitation. Manual cervical dislocation, when properly conducted, is a humane method of euthanasia for smaller animals such as mice. In larger animals, this technique is generally accomplished only by use of mechanical dislocation, owing to a greater muscle mass in the cervical area (1). This method is preferred over other methods because of its ability to induce rapid death while maintaining the brain tissue in a chemically uncontaminated state. However, previous research indicates cervical dislocation can be stressful for the animal and the experimenter. In particular, stress is associated with the physical restraint required to manipulate the conscious animal (2). Furthermore, there are safety concerns to the experimenter (e.g., being bitten) when manipulating a conscious animal prior to physical euthanasia. Data also indicate that electrical activity in the brain persists for 13 sec after cervical dislocation (3), perhaps indicating that pain receptors are activated in the animal. The AVMA Panel on Euthanasia, therefore, recommends that, when practical, animals be rendered unconscious prior to physical euthanasia, using anesthesia.

The rapid anesthetic effects of carbon dioxide (CO_2) are well established (1). In an attempt to ensure a humane method of euthanasia, use of CO_2-induced anesthesia prior to cervical dislocation followed by decapitation has increased recently (2). Controversy regarding the use of anesthesia exists, nevertheless; there is continuing concern about the effects of anesthetics on neurochemical parameters (4). Due to the modicum of research on the effects of CO_2 on brain neurochemistry, the researcher is left with a concern that CO_2 may contaminate neurochemical measures.

Carbon dioxide is a quick-acting and inexpensive anesthetic that does not introduce exogenous chemicals into the body, but works as a CNS depressant drug, increasing respiratory rate until unconsciousness occurs (5, 6). Although the exact mechanism governing CO_2's anesthetic effects is not completely understood, it has been hypothesized that inhalation of CO_2 may induce anesthesia through a narcotic effect and by lowering brain intracellular pH (5). Carbon dioxide-induced anesthesia does not have an effect on cholinergic markers in brain tissue in decapitated rats (7). Furthermore, serum concentrations of tropic hormones in decapitated rats are not adversely affected or increased by CO_2 use prior to euthanasia, indicating that brief exposure to CO_2-induced anesthesia might alleviate the stress of restraint associated with the euthanasia procedure (2). Research has been conducted to date examining the effects of CO_2-induced anesthesia on measurement of biogenic amine concentrations in the mouse brain. The aim of the study reported here was to examine these effects, using high-performance liquid chromatography (HPLC) with electrochemical detection, which is a sensitive method for detecting biogenic amines in brain tissue (8). In particular, three neurotransmitters—norepinephrine, dopamine, and serotonin—were evaluated in four commonly studied brain regions: the frontoparietal cortex, hippocampus, striatum, and cerebellum. These regions are the principal areas of localization and innervation from the widespread biogenic amine systems (9, 10).

All animal procedures were approved by the Wellesley College Animal Care and Use Committee. Twelve specific-pathogen-free, 6-month-old female BALB/cByJ mice (The Jackson Laboratory, Bar Harbor, Maine) were studied. Mice were housed in a facility that has animal care and use programs accredited by AAALAC, International. Mice were kept on pine shavings in polycarbonate cages 15 x 26 x 12 cm (individual cages) or 21 x 44 x 15 cm (group cages) with wire lids. Individually and group-housed mice were evenly distributed between the two experimental groups. The animal room was maintained between 20 to 22°C on a 12/12-h light/dark cycle, with lights on at 0600 h. Mice were fed Harlan Teklad rodent food (no. 8640; Harlan Teklad, Madison, Wis.) and water ad libitum.

Mice were randomly assigned to one of two test groups. Each mouse remained in its home cage in the animal room and was brought to another room immediately before euthanasia. Mice of the first group (n = 6) were introduced individually into an acrylic chamber, 29 x 50 x 28 cm, that had been charged for 15 sec with a 70% CO_2/30% O_2 gaseous mixture from a compressed gas cylinder. Mice remained in the chamber for 30 sec or until they lost consciousness. They were then removed from the chamber and, after cervical dis-

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location, were decapitated. The second group of mice (n = 6) were subjected to cervical dislocation and decapitation without prior anesthesia.

The brain was removed rapidly and dissected immediately while on ice. The frontoparietal cortex was dissected bilaterally, followed by the hippocampus, the striatum, and the cerebellum. Dissected specimens were frozen immediately on dry ice; complete dissection took 2 to 3 min. Specimens were stored at -70°C until the time of the HPLC electrochemical detection assay.

Concentrations of the biogenic amines were measured, using HPLC coupled with electrochemical detection (Bioanalytical Systems, Inc., West Lafayette, Ind.). A model 480 HPLC system was used, consisting of a CC-5 flow cell and a PM-80 solvent delivery pump. The chromatographic column was a reverse phase Microsorb-MV C18 column, with an LC4C electronic controller and a glassy carbon electrode (applied voltage: 650 mV; range: 50 nA; filter: 0.1 Hz). To prepare the mobile phase, 9.6 g of sodium acetate, 0.6 ml of phosphoric acid, 10 mg of EDTA, and 275 mg of octyl sulfate were diluted in 500 ml of HPLC grade water. After the solution was thoroughly stirred, 100 ml of acetonitrile was added; the mobile phase was again stirred and brought to a final volume of 1 L. The pH was adjusted to 3.1. The mobile phase was then filtered through a 0.2 μm nylon membrane, and de-gassed under a vacuum.

Biogenic amine external standards, including norepinephrine, dopamine, and serotonin, were prepared to a final concentration of 1.25 x 10^{-7}M in 50 mM perchloric acid ($HClO_3$). An internal standard of 1.25 x 10^{-7}M 3,4-dihydroxybenzylamine (DHBA) was used to monitor sample degradation. All standards were obtained from Research Biochemical International (Natick, Mass.). Brain specimens were removed from -70°C and placed on ice. Ice-cold 50 mM $HClO_3$, which contained 1.25 x 10^{-7}M DHBA, was added to the tissue in a 20 ml/mg ratio. The $HClO_3$ was added to the cerebellar specimens in a 10 ml/mg ratio because of the large size of these specimens. Sonification of the specimens was done for 15 to 20 sec to disrupt the tissue. The homogenate was centrifuged for 15 min at 14,000 X g to precipitate the protein. The supernatant was carefully removed and was transferred into an autosampler microvial. Twenty microliter samples were injected onto the column by use of the autosampler. Data were collected and analyzed, using Gilson 712 System Controller software.

A repeated measures analysis of variance (ANOVA) was performed on the HPLC data for each of the three neurotransmitters, examining treatment as the main effect and regional differences as the repeated measure. The significance level was set at $P < 0.05$.

Norepinephrine concentration did not differ significantly between animals anesthetized with CO_2 prior to decapitation and those decapitated without prior anesthesia ($F[1,10] = 0.155$, $P > 0.05$). Norepinephrine concentration did vary significantly by region, with the highest value appearing in the cortex and the lowest value appearing in the striatum ($F[3,30] = 14.825$, $P < 0.0001$) (Figure 1).

Dopamine values did not differ significantly between animals anesthetized with CO_2 prior to decapitation and those decapitated without prior anesthesia ($F[1,10] = 0.909$, $P > 0.05$). However, dopamine values differed significantly by region ($F[3,30] = 119.446$, $P < 0.0001$) (Figure 2); they were highest in the striatum and virtually absent in other regions.

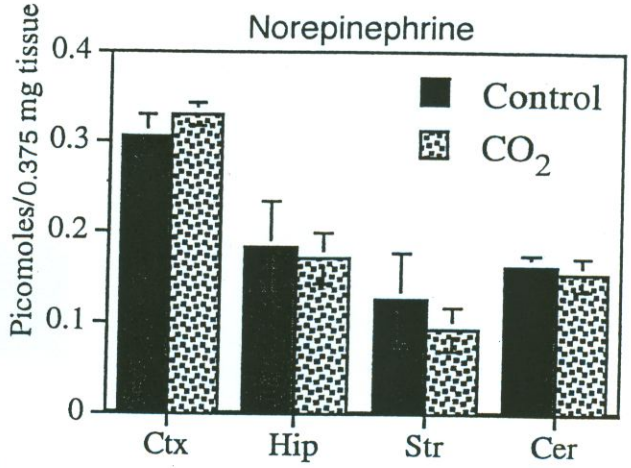

Figure 1. Norepinephrine concentrations(pmoles/0.375 mg tissue) in cortex (Ctx), hippocampus (Hip), striatum (Str), and cerebellum (Cer) after decapitation without prior anesthesia or decapitation after a 30-sec exposure to carbon dioxide. Each bar represents the mean ± SEM for six mice in each group.

Similarly, serotonin values did not differ significantly between animals anesthetized with CO_2 prior to decapitation and those decapitated without prior anesthesia ($F[1,10] = 0.031$, $P > 0.05$), yet serotonin values differed significantly by region ($F[3,30] = 5.207$, $P < 0.01$) (Figure 3). The striatum and the hippocampal regions had higher serotonin values than did the cortical and cerebellar regions.

Minimizing animal pain and distress should always be a consideration in determining the method of euthanasia used. For experiments in which the integrity of the brain tissue is also a concern, the method must also allow for the maintenance of chemically uncontaminated brain tissue. These criteria leave few options for methods of euthanasia. Euthanasia should result in rapid unconsciousness followed by a cessation of cardiac or respiratory activity, which will eventually lead to a loss of brain function. The procedure should minimize anxiety and stress in the conscious animal (1). Cervical dislocation followed by decapitation is a method that is often chosen. However, this method can be stressful to the animal and to the experimenter due to the handling and physical restraint required to perform, and the safety concerns that are associated with, these techniques (1, 2). Furthermore, in educational experiments there could be stress to a student observing this form of euthanasia on a conscious animal, due to its aesthetically displeasing nature. The stress of prolonged restraint can be reduced somewhat by anesthetizing the animal prior to decapitation.

High concentrations of CO_2 can rapidly lead to death in the animal due to mucosal irritation and ventilatory stimulation (11). The AVMA recommends that a CO_2 concentration of 60 to 70% will allow a rapid anesthetic effect while alleviating adverse long-term ventilatory effects (1). In the study reported here, the animals were anesthetized for 30

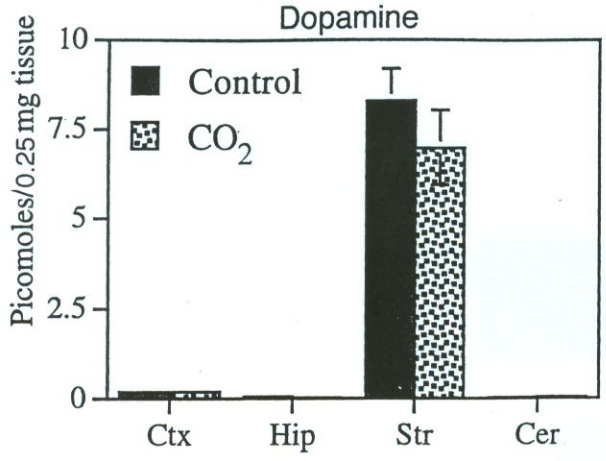

Figure 2. Dopamine concentrations (pmoles/0.25 mg tissue) in various mouse brain regions after decapitation without prior anesthesia or decapitation after a 30-sec exposure to carbon dioxide. Each bar represents the mean ± SEM for six mice in each group. *See* Figure 1 for explanation of abbreviations.

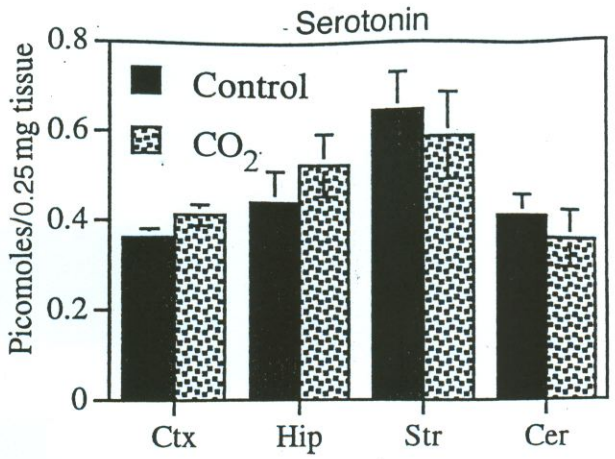

Figure 3. Serotonin concentration (pmoles/0.25 mg tissue) in various mouse brain regions after decapitation without prior anesthesia or decapitation after a 30-sec exposure to carbon dioxide. Each bar represents the mean ± SEM for six mice in each group. *See* Figure 1 for explanation of abbreviations.

sec (until the mice were observed to be immobile) in a precharged chamber with the recommended concentration of 70% CO_2.

The use of CO_2 anesthesia in euthanasia protocols for neurochemical experiments has conventionally been avoided due to concerns that CO_2 anesthesia may alter brain chemistry. Findings from this study indicate that brief exposure to CO_2-induced anesthesia does not significantly alter concentrations of biogenic amines in the mouse brain. Values reported here agree with those previously reported from other studies of brain catecholamine values without CO_2-induced anesthesia (8).

Acknowledgements

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References

1. Report of the AVMA Panel on Euthanasia. 1993. J. Am. Vet. Med. Assoc. **202:**229–249.
2. **Urbanski, H. F., and S. T. Kelley.** 1991. Sedation by exposure to a gaseous carbon dioxide-oxygen mixture: application to studies involving small laboratory animal species. Lab. Anim. Sci. **41:**80–82.
3. **Vanderwolf, C. H., D. P. Buzak, R. K. Cain, *et al.*** 1988. Neocortical and hippocampal electrical activity following decapitation in the rat. Brain Res. **451:**340–344.
4. **Bertorelli, R., A. Hallsotrom, Y. L. Hurd, *et al.*** 1990. Anesthesia effects on in vivo acetylcholine transmission: comparisons of radioenzymatic and HPLC assays. Eur. J. Pharmacol. **175:**79–83.
5. **Freed, D. L. J.** 1983. CO_2 euthanasia. Nature (letter) **304:** 482.
6. **Green, C. J.** 1979. Animal anesthesia. Laboratory Animals, Ltd., London, England.
7. **Berger-Sweeney, J., U. V. Berger, M. Sharma, *et al.*** 1994. Effects of carbon dioxide-induced anesthesia on cholinergic parameters in rat brain. Lab. Anim. Sci. **44:**369–371.
8. **Zaczek, R., and J. T. Coyle.** 1982. Rapid and simple method for measuring biogenic amines and metabolites in brain homogenates by HPLC-electrochemical detection. J. Neural Trans. **53:**1–5.
9. **Koch, D. D., and P. T. Kissinger.** 1980. Liquid chromatography with pre-column sample enrichment and electrochemical detection. Regional determination of serotonin and 5-hydroxyindoleacetic acid in brain tissue. Life Sci. **26:** 1099–1107.
10. **Maruyama, Y., T. Oshima, and E. Nakajima.** 1980. Simultaneous determination of catecholamines in rat brain by reversed-phase liquid chromatography with electrochemical detection. Life Sci. **26:**1115–1120.
11. **Danneman, P. J., S. Stein, and S. O. Walshaw.** 1997. Humane and practical implications of using carbon dioxide mixed with oxygen for anesthesia or euthanasia of rats. Lab. Anim. Sci. **47:**376–385.

Carbon dioxide euthanasia in rats: oxygen supplementation minimizes signs of agitation and asphyxia

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Summary

This paper records the effects of carbon dioxide when used for euthanasia, on behaviour, electrical brain activity and heart rate in rats. Four different methods were used. Animals were placed in a box (a) that was completely filled with carbon dioxide; (b) into which carbon dioxide was streamed at a high flow rate; (c) into which carbon dioxide was streamed at a low flow rate and (d) into which a mixture of carbon dioxide and oxygen was streamed at a fast rate. It was found that the cessation of behaviour was associated with an aberrant pattern of electrical brain activity together with an abnormally low heart rate. The time to reach this point was shortest in those animals placed in the box filled with pure carbon dioxide, longer when carbon dioxide was introduced at a high rate into the box, longer still when oxygen was added to the carbon dioxide gas, and longest when carbon dioxide was streamed slowly into the box. In the condition with pure carbon dioxide, signs of behavioural agitation and asphyxia were seen. This was also true for the two conditions in which carbon dioxide streamed into the box, but to a lesser degree. These signs occurred when some degree of consciousness may still have been present in the animals. Signs of agitation and asphyxia were almost completely absent in the condition where oxygen was added to the carbon dioxide. These results not only demonstrate the usefulness of behavioural criteria next to electrophysiological indices, but also demonstrate that the negative effects of carbon dioxide euthanasia can be prevented by an additional supply of oxygen.

Keywords Euthanasia; rat; carbon dioxide; oxygen; behaviour; electroencephalogram; electrocardiogram

Euthanasia, or the killing of animals in a humane way, is frequently performed in several settings. It is obvious that a procedure should be followed which causes the least possible distress and suffering, while at the same time being safe for the practitioners (AVMA 1963, AVMA 1986). For laboratory animals, methods of inhalation euthanasia involving ether vapour, for example, are not easy to apply safely and are being questioned more and more because of the signs of distress that they produce. In addition gases such as nitrogen, carbon monoxide, nitrous oxide and carbon dioxide are also used (Blackshaw *et al.* 1988, Schlingmann 1989, Woodbury *et al.* 1958). Some gases, such as nitrogen, displace oxygen from the air to be

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breathed whereas others, such as carbon monoxide, prevent binding of oxygen to the haemoglobin in the blood. However, carbon monoxide is seldom used because it is also toxic for those who must administer it. There are also problems with nitrogen because of its lower efficacy in young animals (Glass *et al.* 1944). Gases, such as nitrous oxide, directly affect the central nervous system, whereas carbon dioxide has a 2-fold function: it acts both on the removal of oxygen from the breathing air as well as directly on the central nervous system.

In the last few years, it has become evident that administration of carbon dioxide can be a fast and safe euthanasia method. In particular the induction of unconsciousness is rapid (Forslid 1987, Forslid & Augustinsson 1988). Nevertheless, there are indications that there may be considerable distress in the short period prior to the loss of consciousness (Forslid *et al.* 1986, Freed 1983, Paton 1983). Following administration of pure carbon dioxide in the short time leading up to anaesthesia, pigs exhibited serious signs of asphyxia and gasping (Cantieni 1977, Hoenderken *et al.* 1983). Similar signs were noticed in rats by Van den Bogaard *et al.* (1985). However, these investigators suggested that the negative effects of carbon dioxide could be ameliorated by the addition of oxygen. Under these conditions, animals apparently became unconscious without showing behavioural signs of asphyxia or suffocation, but supplemental parameters to support this observation were lacking.

In this paper, of which an earlier version was published in Dutch (Van Luijtelaar *et al.* 1993), we report on an experiment in which the effects of various euthanasia methods employing carbon dioxide are compared. In one of the conditions, oxygen is added to carbon dioxide in order to establish whether this method indeed results in a more acceptable death than those involving carbon dioxide alone. In addition to behavioural parameters, we obtained measures of the electroencephalogram and electrocardiogram to describe the course of the euthanasia process.

Methods

Twenty-eight male Wistar rats aged between 8 and 13 months served as subjects. They were born and raised under standard conditions in our laboratory, singly housed in Macrolon cages (30 × 25 × 25 cm) with standard rat food and tap water, and maintained on a 12–12 h light-dark cycle with lights on at 10 pm. Under deep pentobarbital anaesthesia, a tripolar electrode set (Plastics One, MS 333/2A) was implanted into all rats over the cortex to measure the electroencephalogram (EEG). Electrodes were placed in the frontal region and in the parietal region with stereotaxic coordinates A 2.0, L 3.5 and A −6.0. L 4.0 respectively (with skull surface flat and bregma 0–0), whereas the earth electrode was placed in the cerebellum.

Animals were allowed to recover from surgery, then they were involved in an experiment for sleep–wake characteristics (Van Luijtelaar *et al.* 1990). This also resulted in extensive habituation to the recording leads and to experimental settings. Some weeks after this investigation, the euthanasia experiment was performed. An animal was taken to a separate room in which no other rats were present. To register the electrocardiogram (ECG), one Aesculap-Michelle clamp was fixed in the skin of the neck and a second one in the skin of the back. Then, the rat was placed in the euthanasia chamber and recordings started.

Animals were randomly allocated to one of 4 conditions ($n = 7$). In the first condition, they were placed in the euthanasia box that was completely filled with carbon dioxide (CO_2–100). In the second condition, rats were put in the box filled with air, but immediately after placement of the animals, carbon dioxide started to stream into the box with a flow rate of 22.5 l/min (CO_2-fast). In the third condition, gas streamed into the box at a slower speed of 2.5 l/min (CO_2-slow). In the fourth condition, the instreaming gas consisted of a mixture of carbon dioxide with oxygen, 22.5 l carbon dioxide and

11.25 l oxygen (CO_2–O_2) per min. The addition of oxygen was restricted to the first 90 sec (Van den Bogaard *et al.* 1985).

The box in which euthanasia took place, was made of glass and had a volume of 18 l (23 × 26 × 33 cm). Humidified gas was introduced in all conditions through a perforated inlet tube at the bottom of the chamber. The chamber was carefully cleaned and dried after finishing each animal.

The EEG (1–70 Hz) and the ECG (1–30 Hz) were recorded throughout the experiment using FETs to prevent movement artifacts, and both signals were written with a speed of 1 cm/sec on chart paper of a polygraph (Elema-Schönander 800). The frequency of the heart rate, based on the R-R interbeat interval, was also recorded. Simultaneously with the electrical registrations, video recordings were made for off-line behavioural analysis. To follow the behavioural changes during the process of euthanasia, 2 independent observers scored the behaviour of the animals from the video recordings. Moreover, these observers also rated the degree to which the animals showed signs of gasping and asphyxia on a 3-point scale. (2 = signs clearly visible; 1 = some signs evident; 0 = no signs evident.)

Conditions were compared using a one-way analysis of variance (ANOVA) with 4 levels of the condition factor. Significant condition differences were followed by Duncan's *post hoc* tests ($P<0.05$) to determine the locus of these differences.

Results

A fixed pattern of 4 phases could be determined in the behaviour of the rats. In Phase I they showed normal, well-coordinated movements of head, body and legs. Most of the time exploratory behaviour was seen. Phase II was characterized by a continuous abnormal high activity, excitation and agitation, with all kinds of behavioural patterns, executed on a higher speed than normally seen. Phase III started when the animal's hindlegs sagged and the rat began to lose

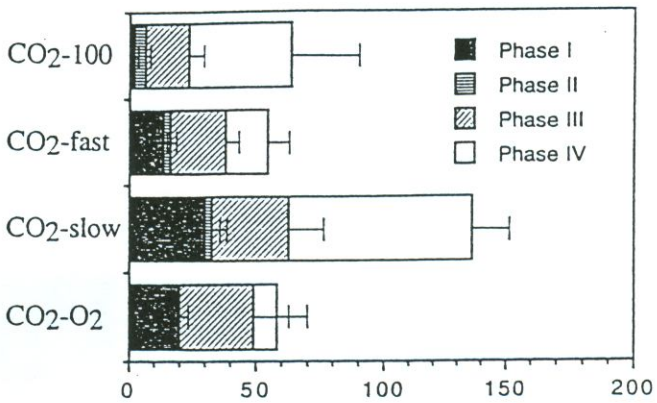

Fig 1 Effects of the carbon dioxide conditions on the phases in behaviour. Mean durations and standard deviations are indicated in seconds ($n=7$). Phase I: normal behaviour; Phase II: excitation phase; Phase III: loss of postural control; Phase IV: complete immobility.

bodily control. This phase ended when postural control was completely lost and muscle tone had totally disappeared. At that time the head sank down and Phase IV began. Except for an occasional twitch or other reflex, the animal lay completely motionless. The phase ended with a last twitch and cessation of respiration.

Behavioural data are shown in Fig. 1. The four euthanasia methods differed in terms of the durations of three phases: for Phase I ($F=62.29$, df 3,26, $P<0.0001$), Phase II ($F=7.05$, df 3,26, $P<0.01$) and Phase IV ($F=5.82$, df 3,26, $P<0.01$). The duration of Phase I, associated with normal behaviour, was shortest in the CO_2–100 group, followed by the CO_2-fast group, which in turn was followed by the CO_2–O_2 group. The duration was longest in the CO_2-slow group. Phase II, characterized by signs of agitation and excitation, was completely absent in the CO_2–O_2 group, while the other conditions did not differ from each other. There were no differences in Phase III, the phase in which the loss of postural control took place. The duration of Phase IV, in which the animal was fully motionless, was longer for the CO_2-slow group compared to the CO_2–O_2 group. The total elapsed time from the beginning of Phase I until the end of Phase IV, was longer for the CO_2-slow

group than for all other groups ($F = 8.03$, df 3,26, $P < 0.001$).

In some conditions it seemed that animals suffered from asphyxia. The animals gasped with their mouths opened wide, and their heads turned up and backward. This kind of forced breathing pattern occurred during Phase II and Phase III. The amount of gasping differed considerably among conditions. Animals in the CO_2–100 group showed most evidence of this behaviour and undoubtedly suffered from serious asphyxia (mean score with SD of the observers: 2.0 ± 0.0). Rats from the CO_2-fast and the CO_2-slow groups also showed this forced breathing pattern, but to a lesser extent and with more variability between animals (mean score with SD: 1.4 ± 0.7; 0.9 ± 0.6 respectively). Animals in the CO_2–O_2 group, however, did not show any signs of asphyxia and were given a score of 0, except for one rat which received a 1 (mean score with SD: 0.1 ± 0.2).

The EEG measures contained a number of characteristic periods that could be recognized and the EEG data are presented in Fig. 2. Before the onset of euthanasia, rats mainly showed an EEG with a pattern of small amplitude, high frequency waves. Following onset of the various conditions, initially no changes could be observed (Period I). Then, in all conditions a further decrease of the amplitude of the overall EEG was noticed. (Period II). This pattern

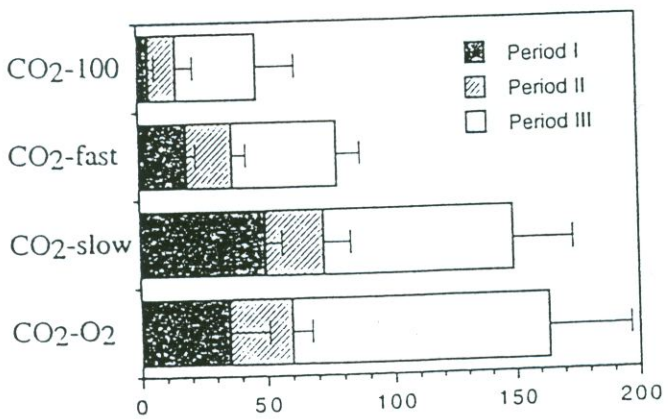

Fig 2 Effects of the carbon dioxide conditions on the periods in the EEG. Mean durations and standard deviations are indicated in seconds ($n = 7$). Period I: normal EEG; Period II: EEG with lowered amplitude; Period III: EEG with slow waves, large spikes and silent phases.

with exceptionally small waves was followed by an EEG containing large, slow waves, often intermingled with high voltage spikes, and always alternated by short periods of silence (Period III). This Period III ended with this heavily fluctuating pattern changed into a complete isoelectric trace. Statistical analysis revealed differences among conditions in the duration of Period I ($F = 32.13$, df 3,22, $P < 0.0001$). Period I was shortest in the CO_2–100 group, followed by the CO_2-fast group, then the CO_2–O_2 group and finally the CO_2-slow group. The duration of Period II, the period with the small amplitude EEG, also differed among groups ($F = 3.18$, df 3,20, $P < 0.05$). This period was shorter for the CO_2–100 group compared to the others. The duration of Period III, with the aberrant EEG, was shorter in the CO_2–100 group and in the CO_2-fast group than in the other groups ($F = 7.36$, df 3,21, $P < 0.01$). Finally, the conditions differed in terms of the time at which the EEG trace became isoelectric ($F = 27.72$, df 3,23, $P < 0.0001$). This moment came later for the CO_2–O_2-slow group than for the CO_2-fast and the CO_2–100 group. Also the total duration of the 3 periods together was shorter in the latter group.

The changes in heart activity could also be classified in 3 stages. In the first tens of seconds the heart rate was quite normal for aroused animals, with frequencies slightly above 300 beats per minute (Stage I). After this period, the heart rate rapidly decreased till an abnormal low value of less than 150 beats per minute. This low heart rate remained stable for a certain time (Stage II) and terminated when the heart started to fibrillate (Stage III). In general, fibrillations preceded the ultimate cessation of the heart. The duration of the three stages which could be recognized in the ECG-registration and the total time before complete cessation of the heart beat, are given in Table 1. The duration of Stage I, with the normal heart rate was longer in the CO_2–O_2 and the CO_2-slow group than in the the CO_2–100 group and the CO_2-fast group ($F = 10.61$, df 3,19, $P < 0.001$). The groups did not differ in the duration of

Table 1 Effects of the carbon dioxide conditions on ECG-recordings. Mean durations and standard deviations for the three stages are indicated in seconds ($n=7$). Stage I: normal heart rate; Stage II: abnormal low heart rate; Stage III: fibrillations till cessation of heart.

	Stage I	Stage II	Stage III	Total time
CO_2–100	28.3±14.7	321.7±91.7	58.3±26.0	408.3±107.1
CO_2-fast	34.2±19.3	297.0±126.1	64.0±23.0	395.2±118.7
CO_2-slow	75.8±16.3	388.8±51.7	66.3±51.7	530.9±74.2
CO_2–O_2	59.0±15.2	451.0±80.8	108.0±52.6	618.0±83.2

Stage II. In fact, in 25 out of 28 animals fibrillations of the heart preceded stopping of the heart. Finally, the cessation of the heart occurred later in the CO_2–O_2 group than in the other 3 conditions ($F=4.87$, df 3,19, $P<0.02$).

Discussion

There were clear differences between the 4 conditions in the durations of the successive behavioural phases that preceded euthanasia. The period with normal behaviour was quite short in the CO_2–100 and considerably longer in the CO_2-slow condition. A further difference was that the phase characterized by complete immobility lasted longer in the CO_2-slow condition compared to the CO_2–100 condition, again supporting the discrepancy between the two extreme conditions. Phase II, the phase with strong behavioural agitation and excitation, was present in the two conditions with instreaming carbon dioxide, and especially in the CO_2–100 condition. This excitation-phase did not exist at all in the CO_2–O_2 condition. Signs of asphyxia were most conspicuous in the CO_2–100 condition and less striking in the two carbon dioxide inflow conditions. The addition of oxygen to the carbon dioxide gas further reduced the signs of agitation. The lack of Phase II in the latter condition also suggests that addition of oxygen strongly diminishes or even prevents behaviour excitation and motor agitation. This confirms the observations made by Van den Bogaard *et al.* (1985).

Initially, the EEG of all rats showed a low amplitude high frequency pattern, which is common for alerted and activated animals. After some time, the amplitude of the waves decreased further but before the onset of isoelectricity, a period occurred in which large and often sharp waves alternated with short phases of isoelectricity. Such a low amplitude EEG is also found in cats and pigs in the beginning phase of anaesthesia with carbon dioxide (Balzamo *et al.* 1991, Forslid 1987). It is also mentioned that application of nitrous oxide to patients induces a similar pattern (Yli-Hankala 1990). Given the fact that in normal situations small waves in the EEG indicate alertness and activation, a possible interpretation of the induced very small waves in the EEG is that this is due to an extreme activation or to a heavy excitation caused by the stressful situation. It may also be associated with a diminished or narrowed consciousness, because the EEG pattern is strongly similar to that at the beginning of an anaesthesia (Coenen &Vendrik 1972). Nevertheless, it remains unclear whether the animal is still conscious during this particular period and, if so, how much distress is experienced. The large waves and spikes in the EEG preceding isoelectricity are easier to understand. Just before an animal dies by an overdose of anaesthetics, muscle twitches and sometimes convulsions can be observed together with large waves and spikes. This is a well-known phenomenon and strong evidence exists for the view that perception and consciousness are then eliminated (Woodbury *et al.* 1958).

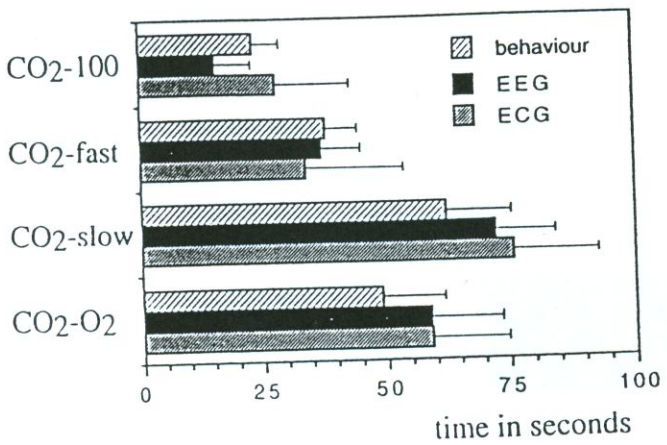

Fig 3 Correspondence between parameters of behaviour, EEG and ECG. For behaviour the time is indicated until complete immobility is reached (Phase I, Phase II and Phase III). For the EEG the time is indicated till the pattern is fully aberrant (Period I and Period II) and in case of the ECG the time is indicated till the heart rate reached an abnormal low rate (Stage I). Mean durations and standard deviations are given in seconds. Within the 4 groups there are no differences among the durations of the 3 parameters

The duration of Stage I of the heart rate, in which the frequency varies between normal levels of 300 and 350 beats per minute (DiCara & Miller 1969), is longest for the CO_2-slow and the CO_2–O_2 conditions. Stages II and III last for a relatively long time and due to a large variance, no differences between conditions were detected, an exception being the cessation of the heart beat that occurs later in the CO_2–O_2 condition.

It is striking that the sudden and large decrease of the frequency of the heart rate at the end of Stage I coincides with the onset of complete behavioural immobility at the end of Phase II of behaviour. Interestingly, these two phenomena are associated with the appearance of a totally disturbed EEG-pattern at the start of Period III. This correlation between immobility, aberrant EEG and abnormal low heart rate, which is shown in Fig. 3, supports and facilitates our view that the loss of consciousness is completed by that time. It also means in our opinion that until that moment the level of discomfort should be minimized.

It is against this background that we interpret our results. The induction of euthanasia with pure carbon dioxide is a fast process, but during the induction animals show signs of serious asphyxia and behavioural excitation. These manifestations of distress occur during the period when the EEG is normal as well as during the period in which the EEG shows a reduced amplitude. Animals begin to suffocate in these periods, at a time when it is not clear whether they have fully lost consciousness. The condition with the fast instreaming carbon dioxide is almost similar to the pure carbon dioxide condition, but the amount of asphyxia is slightly less. Rats in the condition with the slow inflow of carbon dioxide, still show some signs of asphyxia although with less behavioural excitation, but it takes longer before the animals are completely immobile and unconscious. In the condition in which carbon dioxide is mixed with oxygen almost no signs of asphyxia and excitation can be observed. In the latter group, the main action of the carbon dioxide is not its suffocating activity, but, because of the addition of oxygen, its anaesthetic activity. Suffocation and anoxia occur after the onset of complete anaesthesia.

If we judge the present findings according to the criteria for an acceptable procedure for euthanasia, as formulated in 1986 by the 'Panel on Euthanasia' of the American Veterinary Medical Association (1986), we can conclude that the important criterion of minimal discomfort is best satisfied by the condition in which oxygen is added to carbon dioxide. Another criterion refers to the time that elapses till the moment of unconsciousness and death. This time must be as short as possible. According to this criterion, the pure carbon dioxide condition would be judged to be the most suitable. It is clear, then, that these two important criteria are in conflict, and since a choice has to be made between a fast death, with the possibility of asphyxia and stress can be experienced, and a slower but presumably less stressful death, we give priority to the criterion of minimal

discomfort over that of speed. This implies that we prefer the mixture of carbon dioxide and oxygen for inhalation euthanasia. When euthanasia is induced with such a mixture, signs of discomfort are minimal in a period during which the possibility of consciousness cannot be fully excluded.

References

American Veterinary Medical Association (AVMA) (1963) Report of the A.V.M.A. Panel on Euthanasia. *Journal of the American Veterinary Medical Association* **142**, 162–4

American Veterinary Medical Association (AVMA) (1986) Report of the A.V.M.A. Panel on Euthanasia. *Journal of the American Veterinary Medical Association* **188**, 252–68

Balzamo E, Gayan-Ramirez G, Jammes Y (1991) Quantitative EEG changes under various conditions of hyperventilation in the sensorimotor cortex of the anaesthetized cat. *Electroencephalography and Clinical Neurophysiology* **78**, 159–65

Blackshaw JK, Fenwick DC, Beattie AW, Allan DJ (1988) The behaviour of chickens, mice and rats during euthanasia with chloroform, carbon dioxide and ether. *Laboratory Animals* **22**, 67–75

Cantieni J (1977) Ein Beitrag zur CO_2-Betäubung von Schlachtschweinen. *Schweizerische Archive der Tierheilkunde* **119**, 355–75

Coenen AML, Vendrik AJH (1972) Determination of the transfer ratio of cat's geniculate neurons through quasi-intracellular recordings and the relation with the level of alertness. *Experimental Brain Research* **14**, 227–42

DiCara LV, Miller NE (1969) Heart rate learning in the noncurarized state, transfer to the curarized state, and subsequent retraining in the noncurarized state. *Physiology and Behavior* **4**, 621–4

Forslid A (1987) Transient neocortical, hippocampal and amygdaloid EEG silence induced by one minute inhalation of high concentration CO_2 in swine. *Acta Physiologica Scandinavica* **130**, 10

Forslid A, Augustinsson O (1988) Acidosis, hypoxia and stress hormone release in response to one-minute inhalation of 80% CO_2 in swine. *Acta Physiologica Scandinavica* **132**, 223–31

Forslid A, Ingvar M, Rosén I, Ingvar DH (1986) Carbon dioxide narcosis: influence of short-term high concentration carbon dioxide inhalation on EEG and cortical evoked responses in the rat. *Acta Physiologica Scandinavica* **127**, 281–7

Freed DLJ (1983) CO_2 euthanasia. *Nature* **304**, 482

Glass HG, Snyder FF, Webster E (1944) The rate of decline in resistance to anoxia of rabbits, dogs, and guinea pigs from the onset of variability to adult life. *American Journal of Physiology* **140**, 609–15

Hoenderken R, Van Logtestijn JG, Sybesma W, Spanjaard WJM (1979) Kohlendioxid-Betäubung von Schlachtschweinen. *Die Fleischwirtschaft* **59**, 1572–8

Paton WDN (1983) Is CO_2 euthanasia humane? *Nature* **305**, 268

Schlingmann F (1989) Rotor voor het op verantwoorde wijze doden van grote aantallen kleine laboratoriumdieren. *Biotechniek* **28**, 38–41

Van den Bogaard AEJM, van Dam E, Weekers FH (1985) Het gebruik van een koolzuurgas apparaat voor ratten. *Biotechniek* **24**, 34–8

Van Luijtelaar G, Drinkenburg P, Hoederken R, Coenen A (1993) Euthanasie met koolzuurgas bij de rat: Effecten van het toevoegen van zuurstof. *Biotechniek* **32**, 46–50

Van Luijtelaar ELJM, Stephens DN, Coenen AML (1990) Comparison of abecarnil and diazepam on hypnotic activity in rats. In: *Sleep '90* (Horne J, ed.) Bochum: Pontenagel Press, pp 152–4

Woodbury DM, Rollins LT, Gardner MD, *et al.* (1958) Effects of carbon dioxide on brain excitability and electrolytes. *American Journal of Physiology* **192**, 79–89

Yli-Hankala A (1990) The effect of nitrous oxide on EEG spectral power during halothane and isoflurane anaesthesia. *Acta Anaesthesiologica Scandinavica* **34**, 579–84