

PDF hosted at the Radboud Repository of the Radboud University Nijmegen

The following full text is a publisher's version.

For additional information about this publication click this link.

<http://hdl.handle.net/2066/29054>

Please be advised that this information was generated on 2018-07-07 and may be subject to change.

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

A. M. L. Coenen¹, W. H. I. M. Drinkenburg¹, R. Hoenderken² &
E. L. J. M. van Luijelaar¹

¹NICI, Department of Psychology, University of Nijmegen, PO Box 9104, 6500 HE Nijmegen, and

²Ministry of Welfare, Health and Culture, Veterinary Public Health Inspectorate, PO Box 9013,
6800 DR Arnhem, The Netherlands

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

Correspondence to: Dr A. M. L. Coenen

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 (CO₂-100). 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 (CO₂-fast). In the third condition, gas streamed into the box at a slower speed of 2.5 l/min (CO₂-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 ($\text{CO}_2\text{-O}_2$) 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 ($23 \times 26 \times 33$ 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

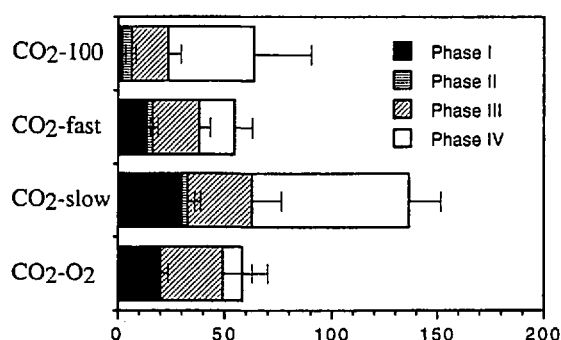


Fig 1 Effects of the carbon dioxide conditions on the phases in behaviour. Mean durations and standard deviations are indicated in seconds ($n = 7$). Phase I: normal behaviour; Phase II: excitation phase; Phase III: loss of postural control; Phase IV: complete immobility.

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 $\text{CO}_2\text{-100}$ group, followed by the $\text{CO}_2\text{-fast}$ group, which in turn was followed by the $\text{CO}_2\text{-O}_2$ group. The duration was longest in the $\text{CO}_2\text{-slow}$ group. Phase II, characterized by signs of agitation and excitation, was completely absent in the $\text{CO}_2\text{-O}_2$ 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 $\text{CO}_2\text{-slow}$ group compared to the $\text{CO}_2\text{-O}_2$ group. The total elapsed time from the beginning of Phase I until the end of Phase IV, was longer for the $\text{CO}_2\text{-slow}$

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

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 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

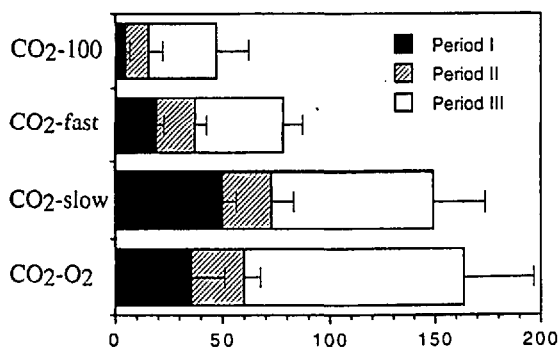


Fig 2 Effects of the carbon dioxide conditions on the periods in the EEG. Mean durations and standard deviations are indicated in seconds ($n = 7$). Period I: normal EEG; Period II: EEG with lowered amplitude; Period III: EEG with slow waves, large spikes and silent phases.

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 CO₂-O₂ 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 CO₂-100 and considerably longer in the CO₂-slow condition. A further difference was that the phase characterized by complete immobility lasted longer in the CO₂-slow condition compared to the CO₂-100 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 CO₂-100 condition. This excitation-phase did not exist at all in the CO₂-O₂ condition. Signs of asphyxia were most conspicuous in the CO₂-100 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).

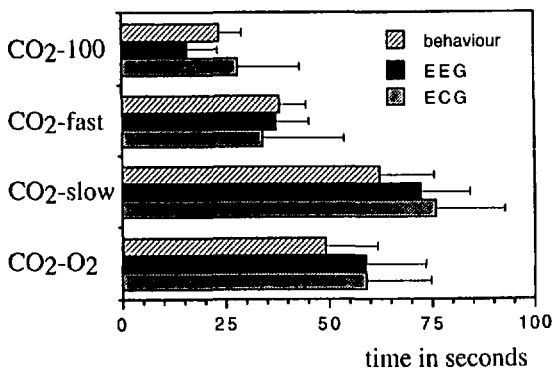


Fig 3 Correspondence between parameters of behaviour, EEG and ECG. For behaviour the time is indicated until complete immobility is reached (Phase I, Phase II and Phase III). For the EEG the time is indicated till the pattern is fully aberrant (Period I and Period II) and in case of the ECG the time 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 CO₂-slow and the CO₂-O₂ 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 CO₂-O₂ 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 CO₂-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 CO₂ 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% CO₂ 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 DL (1983) CO₂ 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 CO₂ 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 Luijteleaer G, Drinkenburg P, Hoenderken R, Coenen A (1993) Euthanasie met koolzuurgas bij de rat: Effecten van het toevoegen van zuurstof. *Biotechniek* **32**, 46-50
- Van Luijteleaer 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