Carbonic anhydrase and control of breathing: different effects of benzolamide and methazolamide in the anaesthetized cat

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1. The effect of inhibition of erythrocyte carbonic anhydrase on the ventilatory response to CO_2 was studied by administering benzolamide (70 mg kg^{-1}, I.V.), an inhibitor which does not cross the blood–brain barrier, to carotid body denervated cats which were anaesthetized with chloralose–urethane.

2. In the same animals the effect on the ventilatory response to CO_2 of subsequent inhibition of central nervous system (CNS) carbonic anhydrase was studied by infusing methazolamide (20 mg kg^{-1}), an inhibitor which rapidly penetrates into brain tissue.

3. The results show that inhibition of erythrocyte carbonic anhydrase by benzolamide leads to a decrease in the slope of the normoxic CO_2 response curve, and a decrease of the extrapolated arterial P_{CO_2} at zero ventilation.

4. Inhibition of CNS carbonic anhydrase by methazolamide results in an increase in slope and x-intercept of the ventilatory CO_2 response curve.

5. Using a mass balance equation for CO_2 of a brain compartment, it is argued that inhibition of erythrocyte carbonic anhydrase results in a decrease in slope of the *in vivo* CO_2 dissociation curve, which can explain the effects of benzolamide.

6. The changes in slope and intercept induced by methazolamide are discussed in relation to effects on neurones containing carbonic anhydrase, which may include central chemoreceptors.

In man and other mammalian species, carbonic anhydrase is ubiquitously present throughout the body. Examples of peripheral tissues and cells containing distinct isoenzymes are erythrocytes, carotid bodies, striated (mainly type I) muscles, lung capillary endothelium, renal tubular cells and other cells of secretory organs such as the choroid plexus (Lee & Mattenheimer, 1964; Maren, 1967; Effros, Chang & Silverman, 1978; Dodgson, 1991; Geers & Gros, 1991). Within the central nervous system (CNS) carbonic anhydrase is mainly present in oligodendrocytes but apparently also in neurones, for example in the rostroventrolateral medulla oblongata where central chemoreceptors are thought to be located (Giacobini, 1962; Ridderstråle & Hanson, 1985). Most of these structures are involved, directly or indirectly, in the neural control of breathing or in the rate at which CO_2 molecules and H^+ ions are removed from the body. As a consequence, simultaneous inhibition of carbonic anhydrase in all these structures will result in a respiratory effect consisting of different contributions of distinct tissues containing carbonic anhydrase.

Selective carbonic anhydrase inhibition at only one site would be a suitable means to study the contribution of the enzyme at that site to the control of breathing. In this study we focus on the role of erythrocyte and brain carbonic anhydrase. In most studies on the respiratory effects of inhibitors of the enzyme the sulphonamide, acetazolamide, has been used (see reviews by Maren, 1967, and Swenson, 1984). After oral or intravenous administration, however, this drug is unevenly distributed, resulting in an accumulation in secretory tissues and a relative, but not absolute, exclusion from the brain (Roth, Schoolar & Barlow, 1959; Maren, 1967, 1977). This implies that in order to achieve a full physiological effect of brain carbonic anhydrase inhibition with acetazolamide in an acute study, which demands more than 99% inhibition, unacceptably large doses are required (Maren, 1967, 1977; Hanson, Nye & Torrance, 1981). Furthermore, at the same time, erythrocyte, carotid body and renal carbonic anhydrase would also be effectively inhibited, complicating an interpretation of a ventilatory effect of the drug.

Methazolamide does not have the disadvantages of acetazolamide: the drug is more water and lipid soluble and diffuses much better into tissues. In contrast to acetazolamide, it is evenly distributed and penetrates easily into the brain (Maren, 1977). Benzolamide, another sulphonamide carbonic anhydrase inhibitor, has quite opposite properties: its lipid solubility and diffusivity are

very much lower (Holder & Hayes, 1965). Furthermore, its plasma binding is much higher than that of methazolamide, leaving less unbound molecules available to traverse membranes (Holder & Hayes, 1965; Maren, 1967). At physiological pH, 99% of the drug is ionized, compared with 61% for methazolamide. Due to these properties, benzolamide only crosses the blood–brain barrier very slowly. From data obtained in rats and dogs (Travis, Wiley & Maren, 1966) it seems very unlikely that, after a single intravenous infusion of less than 100 mg kg^{-1}, a significant quantity of benzolamide will have entered the brain within 4 h. A consequence of the different properties of benzolamide and methazolamide is that if they are given in this order, the former can be used to study the role of carbonic anhydrase in peripheral tissue(s), and the latter to block the enzyme in the CNS.

The two purposes of the present study in cats were: (1) to study the effects on the control of breathing of selective inhibition of erythrocyte carbonic anhydrase by intravenously administered benzolamide; and (2) to study the ventilatory response to CO_2 of subsequent inhibition of CNS carbonic anhydrase by methazolamide.

A contribution of the carotid bodies to possible ventilatory effects was prevented by bilateral section of the carotid sinus nerves. Respiratory effects of metabolic acidosis due to inhibition of renal carbonic anhydrase were prevented by infusing as much bicarbonate as was necessary to maintain a constant base excess.

METHODS

Eight adult cats (weight 2·5–4·5 kg) were sedated with 15 mg kg^{-1} ketamine hydrochloride. Atropine sulphate (0·5 mg s.c.) was given. The animals were anaesthetized with gas containing 0·5–1% halothane and 30% O_2 in N_2 while the femoral arteries and veins were cannulated. Subsequently 20 mg kg^{-1} α-chloralose and 100 mg kg^{-1} urethane were slowly infused intravenously, and the addition of halothane to the inspirate was discontinued. Anaesthesia was maintained with a continuous infusion of 1 mg kg^{-1} h^{-1} α-chloralose and 5 mg kg^{-1} h^{-1} urethane. Rectal temperature was monitored with a thermistor and maintained within 0·5 °C in the range 36·7–38·0 °C by a heating blanket and an infrared lamp.

The trachea was cannulated at midcervical level and connected to a respiratory circuit. Both carotid sinus nerves were identified at their junctions with the glossopharyngeal nerves, and were cut. To check the effectiveness of carotid nerve section, the animals were exposed to a short hypoxic challenge. All cats responded with a decrease in ventilation, indicating that the peripheral chemoreceptors were functionally eliminated.

The animals were connected to an extracorporeal circuit (ECC) receiving blood from the left femoral artery, which was pumped back via the right femoral vein with a flow of 6 ml min^{-1}. In the extracorporeal circuit cuvettes with thermostats were placed for continuous measurement of arterial pH and P_{CO_2}.

Measurements

Respiratory airflow was measured with a Fleisch No. 0 flow transducer (Fleisch, Lausanne, Switzerland) connected to a differential pressure transducer (Statham PM197, Los Angeles, CA, USA), and was electrically integrated to yield tidal volume. The CO_2 and O_2 concentrations in the tracheal gas were measured with an infrared analyser (Gould Godard MK2 Capnograph, Bilthoven, The Netherlands) and a fast-responding zirconium oxide cell (Jaeger O_2-test, Würzburg, Germany), respectively. The respiratory gas concentrations were regulated with mass flow controllers (type AFC 260, Advanced Semiconductor Materials, De Bilt, The Netherlands).

Arterial pH and P_{CO_2} in the blood passing through the extracorporeal circuit were measured continuously with a pH electrode (Radiometer E-5037-0, Copenhagen, Denmark), calibrated with phosphate buffers, and a CO_2 electrode (General Electric A312AB, Milwaukee, WI, USA) which was calibrated with water equilibrated with CO_2–O_2–N_2 gas mixtures delivered by a gas-mixing pump (Wösthoff, Bochum, Germany). Arterial P_{O_2} was measured with a home-made Clarke-type oxygen electrode (outer diameter 1 mm), which was positioned within the cannula of the left femoral artery, very close to the point from which the blood was withdrawn from the animal. By exchanging this P_{O_2} electrode for a P_{CO_2} electrode, we measured in three cats arterial P_{CO_2} at two different sites in the extracorporeal circuit: one '*in vivo*' site close to the animal with a circulatory delay from the lungs between 7 and 12 s (P_{lv,CO_2}), and a second (remote) one with a delay varying between 40 and 57 s (P_{a,CO_2}). The CO_2 electrodes were recalibrated about every 2 h, and corrections were made for drift when necessary (Olievier, Berkenbosch & Quanjer, 1978). Arterial blood pressure was measured using a Statham pressure transducer.

All signals were recorded on polygraphs, digitized (sample frequency 100 Hz), processed by a PDP 11/23 computer (Digital Equipment Corp., Maynard, IO, USA) and stored on disk. Steady-state values of ventilation, blood pressure, end-tidal and arterial blood gas tensions were averaged over twenty breaths.

Drugs

Benzolamide (obtained from a generous gift from Professor T. H. Maren, Gainesville, FL, USA) and methazolamide (purchased from Lederle) were dissolved in a NaOH solution with the pH adjusted to between 7 and 8 with HCl. The drugs were infused intravenously at a rate of about 1 ml min^{-1} in volumes ranging from 4 to 7 ml.

Experimental design

After surgery and connecting the animal to the extracorporeal circuit, about 1 h was allowed for stable conditions to be reached. The cats were breathing spontaneously. All experiments were performed in normoxia (end-tidal O_2 pressure, P_{ET,O_2}, 15 kPa). End-tidal CO_2 was set at different levels and steady-state ventilation was determined. Thereafter, 70 mg kg^{-1} benzolamide was infused intravenously, about 2 h later followed by infusion of 20 mg kg^{-1} methazolamide. With each drug, the ventilatory response it gave as it was being injected was analysed, and then the steady-state responses to changes in P_{a,CO_2} were determined.

Because we continuously measured both the equilibrium P_{a,CO_2} and arterial pH (pH_a) in the extracorporeal circuit, we were able to monitor the acid–base status of the animals at each steady-state

level of end-tidal P_{CO_2} by inspection of the $\log P_{a,CO_2}$–pH_a bufferline. A deviation from this line indicating metabolic acidosis was corrected by infusion of bicarbonate. The drug infusions were made at a constant P_{a,CO_2} (maintained until approximately 60 min after the infusion) in the ECC by manipulating the inspiratory CO_2 fraction. Perturbations in pH_a due to the injectate or to a renal effect of the drugs could be corrected by infusing bicarbonate when necessary (e.g. see Fig. 1).

Data analysis

The steady-state ventilation as a function of P_{a,CO_2},

$$\dot{V}_I = S(P_{a,CO_2} - B),$$

was fitted by linear regression where S is the slope and B the extrapolated P_{a,CO_2} at zero ventilation (x-intercept). In the control situation, the mean range of P_{a,CO_2} over which the ventilatory response was tested, was 5·2–7·0 kPa. After administration of the inhibitors, the mean range of (equilibrium) P_{a,CO_2} was 5·4–7·4 kPa. Within these ranges, the ventilatory responses were linear.

Statistical analysis

The steady-state data were analysed applying a two-way analysis of variance using a fixed model. Differences between the three treatments (control, benzolamide and methazolamide) were tested pairwise with the method of least significant differences. Type I errors were controlled by choosing the level of significance at a probability of $\leq 0{\cdot}01$. Results are presented as means ± S.D. unless otherwise stated.

The design of this study and the use of cats were approved by the Ethical Committee for Animal Experiments of the University of Leiden.

RESULTS

During the experiments the flow rate in the extracorporeal circuit was set at 6 ml min^{-1}. At this rate, the circulatory delay from the lungs to the remote CO_2 electrode measuring P_{a,CO_2} was in the range between 40 and 57 s. After administration of benzolamide a large $P_{(a-ET),CO_2}$ gradient (i.e. the P_{CO_2} gradient between the arterial blood in equilibrium and end-tidal gas) developed: since the speed of the uncatalysed conversion of bicarbonate to CO_2 is much slower than lung capillary transit time, the chemical reaction proceeds after the blood has left the pulmonary capillaries. This implies that at any site within the arterial tree the level of the P_{CO_2} depends on its circulatory delay from the lungs. This is true as long as equilibrium is not yet reached. During inhibition of erythrocyte carbonic anhydrase, the '*in vivo*' P_{CO_2} (P_{iv,CO_2}) was lower than the P_{CO_2} measured by the remote electrode in the ECC (P_{a,CO_2}). This means that at the '*in vivo*' site CO_2 and bicarbonate had not yet reached equilibrium (see also below). When we increased the circulation time from the lungs to the remote electrode in the ECC by decreasing the flow rate to

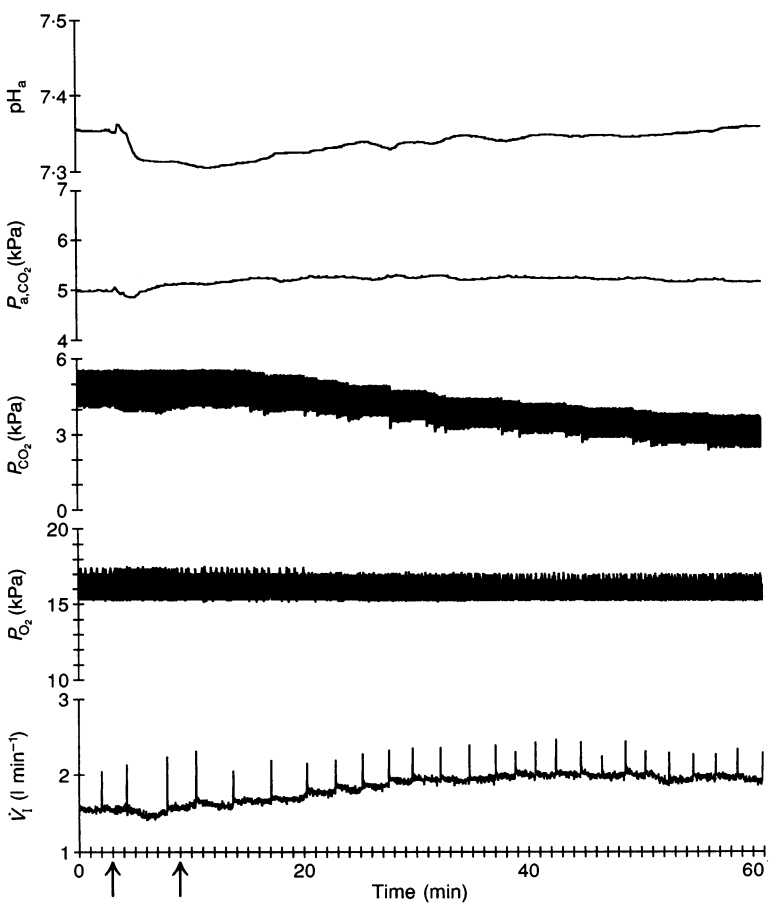

Figure 1. Respiratory effects of 70 mg kg^{-1} benzolamide (I.V.)

Recording of the effect of an intravenous infusion of 70 mg kg^{-1} benzolamide in a spontaneously breathing cat. The arrows indicate start and end of the infusion, respectively. The P_{CO_2} in the extracorporeal circulation (P_{a,CO_2}) was kept at about 5 kPa by withdrawing CO_2 from the inspiratory air. The pH of the arterial blood (pH_a) was brought back to the control value by infusing bicarbonate. Note that full development of the gradient between P_{a,CO_2} and P_{ET,CO_2} took almost 1 h. P_{CO_2} and P_{O_2} are partial pressures in tracheal gas.

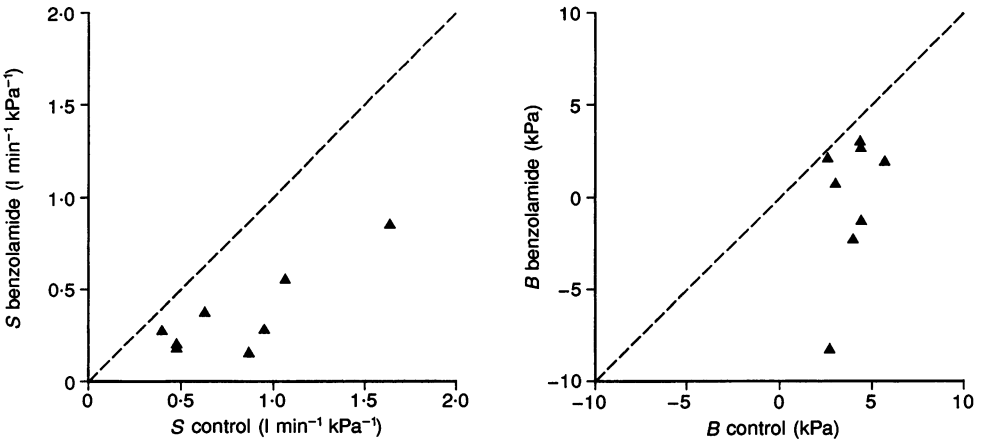

Figure 2. Effects of benzolamide on the ventilatory response curve
Scatter diagrams of the slopes (S) and x-intercepts (B) before and after benzolamide administration.

3 ml min^{-1}, the P_{a,CO_2} at this site did not change, indicating that at 6 ml min^{-1} the measured P_{a,CO_2} was indeed the equilibrium P_{CO_2}: otherwise the remote electrode should have read higher values at lower flow rates.

Generally, the infusion of benzolamide resulted in an inhibition of renal carbonic anhydrase developing within 30–60 min (see also Travis, Wiley, Nechay & Maren, 1964). Since the equilibrium P_{a,CO_2} was kept constant, a tendency for the equilibrium pH_a to decrease (indicating a developing metabolic acidosis) was easily observed and corrected by slowly infusing bicarbonate as long as was necessary (generally 45–60 min).

Ventilatory effects of benzolamide

An example of the effect of an intravenous infusion of 70 mg kg^{-1} benzolamide is shown in Fig. 1. The recording shows that, while P_{a,CO_2} was kept constant at about 5 kPa, ventilation increased. In this example the arterial pH initially fell, probably due to the relatively low pH of the injectate. By infusing molar bicarbonate (mean rate 0·1 ml min^{-1} during 60 min) the pH was brought back to the control value. Note that, after the infusion of benzolamide, full development of the $P_{(a-ET),CO_2}$ gradient took about 45 min. Therefore, in all animals we measured the ventilatory response to CO_2 after about 1 h.

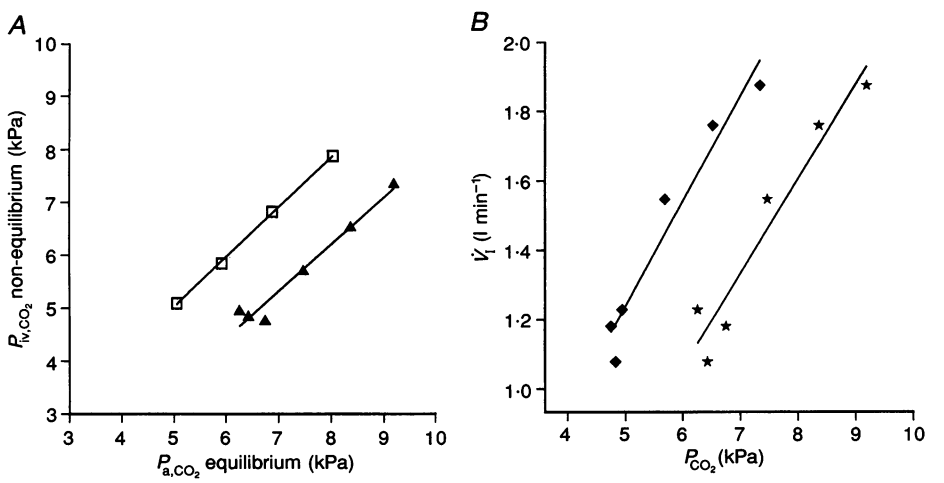

Figure 3. Equilibrium and *in vivo* values of arterial P_{a,CO_2} during inhibition of erythrocyte carbonic anhydrase
A, relationship between the *in vivo* non-equilibrium P_{CO_2} (P_{iv,CO_2}) and equilibrium P_{a,CO_2} in one animal before (□) and after (▲) benzolamide administration. In the control situation the line relating the P_{iv,CO_2} and P_{a,CO_2} was given by: $P_{iv,CO_2} = 0{\cdot}95P_{a,CO_2} + 0{\cdot}27$. After benzolamide infusion the relationship obtained by linear regression was given by: $P_{iv,CO_2} = 0{\cdot}89P_{a,CO_2} - 0{\cdot}92$. The drawn lines are regression lines. *B*, ventilatory CO_2 response curves. The slope of the 'non-equilibrium' regression line (◆) is 0·30 l min^{-1} kPa^{-1}. The equilibrium line (★) has a slope of 0·27 l min^{-1} kPa^{-1}.

The effects of benzolamide on the slope and intercept of the steady-state $\dot{V}_I$–P_{a,CO_2} response curves in eight animals are shown in Fig. 2. As can be seen, after benzolamide the slope as well as the value of B was lower in all animals. The effects of benzolamide are summarized in Table 1. For convenience we denote the parameters after administration by a subscript $_b$. The control slope (S) over S_b averaged 2·28. The mean decrease in B was 4·1 kPa.

After benzolamide infusion, base excess and the $\log P_{a,CO_2}$–pH_a bufferline were not significantly different from control indicating that the bicarbonate infusion had achieved its objective. Benzolamide infusion resulted in a $P_{(a-ET),CO_2}$ gradient of 3·07 ± 0·57 kPa, measured at different levels of end-tidal CO_2.

In three cats the catheter P_{O_2} electrode was replaced by a CO_2 electrode of the same type as was used in the extracorporeal circuit. The tip of the electrode was placed close to the point where blood from the femoral artery left the animal. In all three cats examined, after benzolamide infusion the P_{CO_2} at this *in vivo* site (P_{Iv,CO_2}) was lower than the value read by the remote electrode implying that equilibrium had not yet been reached. The circulatory delay from the lungs to this *in vivo* electrode, determined by applying step changes in end-tidal P_{CO_2}, was 7·8 ± 1·2 s ($n = 15$). The lung-to-brainstem circulation time in the cat is also about 7 s (Teppema, Vis, Evers & Folgering, 1982). The relation between P_{Iv,CO_2} and P_{a,CO_2} was linear with a mean slope of 0·97 and an intercept of −0·6 kPa. Therefore, the slopes of the $\dot{V}_I$–P_{Iv,CO_2} and $\dot{V}_I$–P_{a,CO_2} response curves were about the same, so that the former curve was displaced approximately in parallel to the left. This is illustrated in Fig. 3. We can also infer from these data that the P_{Iv,CO_2} which we measured during inhibition of erythrocyte carbonic anhydrase, was, to a fair approximation, equal to the arterial P_{CO_2} of the blood entering the brainstem, which is about 0·6 kPa lower than the arterial P_{CO_2} at which equilibrium is reached.

Ventilatory effects of methazolamide after prior infusion of benzolamide

An example of the effect of an infusion of 20 mg kg^{-1} methazolamide about 2 h after prior infusion of benzolamide is shown in Fig. 4. Usually a decrease in ventilation at about constant P_{a,CO_2} was observed. The effects of methazolamide on the $\dot{V}_I$–P_{a,CO_2} response curve are shown in the scatter diagram of Fig. 5. After infusion of the drug, significant increases in S and B were found.

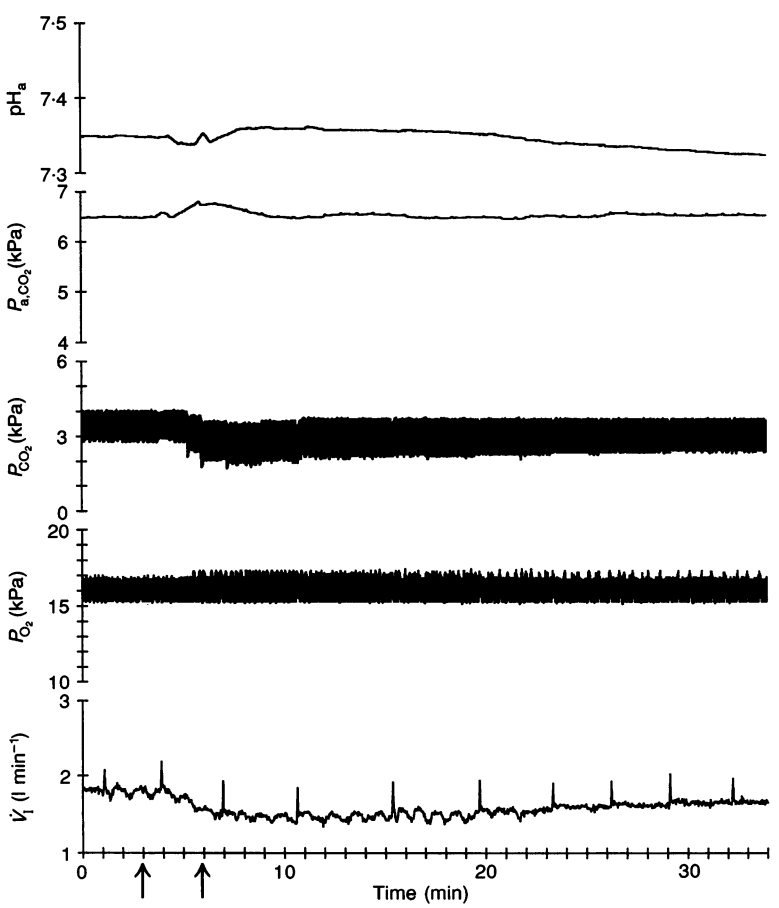

Figure 4. Respiratory effects of 20 mg kg^{-1} methazolamide (I.V.)
Recording of the effects of an infusion of 20 mg kg^{-1} methazolamide (arrows) in a spontaneously breathing cat after prior inhibition of erythrocyte carbonic anhydrase by benzolamide (the recording is from the same animal as in Fig. 1). Inspiratory CO_2 was withdrawn in order to maintain a constant P_{a,CO_2}. Arterial pH (pH_a) did not change appreciably after the infusion and no bicarbonate infusion was necessary. P_{CO_2} and P_{O_2} are partial pressures in tracheal gas.

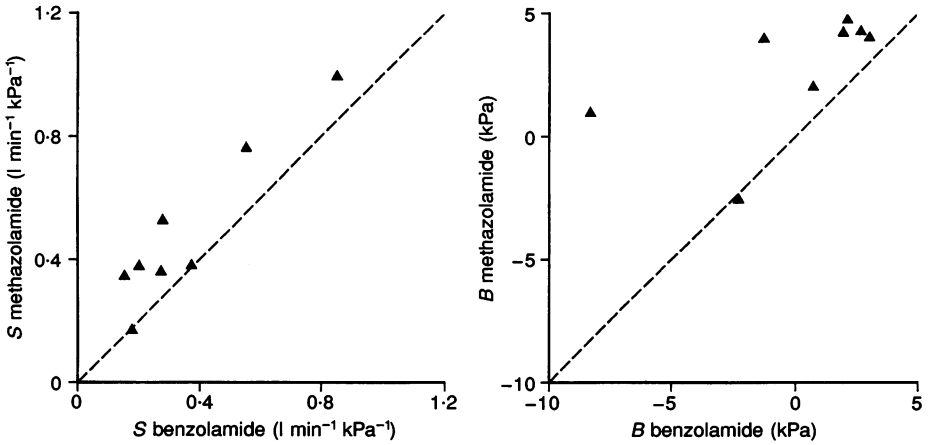

Figure 5. Effects of methazolamide on the ventilatory response curve
Scatter diagrams of the slope (S) and x-intercept (B) of the ventilatory CO_2 response curve before and after methazolamide administration. The animals had received a prior infusion of benzolamide.

The results are summarized in Table 1. The agent produced a statistically insignificant ($P > 0{\cdot}08$) increase of 0·41 kPa in the mean $P_{(a-ET),CO_2}$ gradient (Table 1), contributing little to the relatively large change in mean B of 2·89 kPa.

DISCUSSION

Inhibition of erythrocyte carbonic anhydrase

To achieve an effective inhibition of erythrocyte carbonic anhydrase with benzolamide we administered a dose of 70 mg kg^{-1}. In preliminary studies we had found that intravenous infusion of 50 mg kg^{-1} did not result in a $P_{(a-ET),CO_2}$ gradient or in any ventilatory effect within 30 min. We therefore decided to give a higher dose, in order to be able to compare the results of different treatments within one animal on a reasonable time scale. Even after 70 mg kg^{-1} it took about 1 h before the gradient had fully developed and before a stable condition was reached. Apparently a higher dose of benzolamide is needed to achieve erythrocyte carbonic anhydrase inhibition in cats than in dogs (cf. Travis *et al.* 1964, 1966). The approximately three times higher red cell enzyme activity in cats than in dogs (Dodgson & Forster, 1983) may contribute to this difference. The magnitude of the $P_{(a-ET),CO_2}$ gradient after 70 mg kg^{-1} benzolamide was similar to that found in a previous study after a dose of 50 mg kg^{-1} acetazolamide (Teppema, Rochette & Demedts, 1990), at which erythrocyte carbonic anhydrase is effectively inhibited (see Maren, 1967).

At physiological pH benzolamide exists almost exclusively as a charged molecule, is largely bound to plasma proteins, and has a very low diffusibility (Holder & Hayes, 1965; Maren, 1967). It can be expected therefore that 2–3 h after its administration, a negligible amount of the drug will have crossed the blood–brain barrier with its tight junctions, and that it will be largely excluded from muscles. This is supported by observations of Travis *et al.* (1966) who indeed found very low concentrations of benzolamide in brain and muscle of rats and dogs which were treated with very high daily doses of the drug for as long as 28 days (see also Hanson *et al.* 1981).

Because benzolamide rapidly induces a metabolic acidosis (Travis *et al.* 1964, 1966) which in turn could underlie an

Table 1. Respiratory and acid–base effects of 70 mg kg^{-1} benzolamide (Benz) and of 20 mg kg^{-1} methazolamide (Meth) after prior infusion of benzolamide in carotid body denervated cats

	S (l min^{-1} kPa^{-1})	B (kPa)	$P_{(a-ET),CO_2}$ (kPa)	Base excess (mequiv l^{-1})	Slope bufferline	Intercept bufferline
Control	0·81 ± 0·41	3·89 ± 1·06	0·29 ± 0·36	−4·04 ± 1·28	−1·28 ± 0·17	10·12 ± 1·25
Benz	0·36 ± 0·24*	−0·19 ± 3·78*	3·07 ± 0·57*	−3·85 ± 1·55	−1·25 ± 0·22	9·88 ± 1·58
Meth after Benz	0·49 ± 0·27*†	2·70 ± 2·48†	3·48 ± 0·41*	−3·63 ± 1·39	−1·15 ± 0·15	9·14 ± 1·12

S and B are the slope and x-intercept, respectively, of the line relating minute ventilation and P_{a,CO_2}, which is the P_{CO_2} existing at equilibrium. * Different from control: $P < 0{\cdot}01$. † Different from benzolamide: $P < 0{\cdot}01$. Changes in base excess and in the slopes and intercepts of the bufferlines relating $\log P_{a,CO_2}$ to arterial pH were not allowed to occur after infusion of the drugs (all P values $> 0{\cdot}01$).

effect on ventilation, the base excess was kept constant at the level existing prior to its infusion. Furthermore, the peripheral chemoreceptors of the carotid bodies were functionally eliminated by cutting both carotid sinus nerves. The aortic nerves were left intact because it has been shown that the contribution of the aortic bodies to the ventilatory response to CO_2 changes is of minor importance (Hanson, Rao & Torrance, 1979; Lahiri, Mokashi, Mulligan & Nishino, 1981). Inhibition of erythrocytic carbonic anhydrase means that, within the capillary transit time of about 1 s, much less bicarbonate will be formed in the blood while it perfuses the tissues because the uncatalysed CO_2 hydration reaction takes 10–20 s to reach equilibrium (Maren, 1967). Because normally CO_2 is mainly transported as bicarbonate, this will result in a decrease in slope of the *in vivo* CO_2 dissociation curve of the blood perfusing the tissues, despite the larger contributions of physically dissolved CO_2 and carbamino–Hb, respectively, to the transport of CO_2 (see Cain & Otis, 1961). During inhibition of carbonic anhydrase, the resulting slope of the *in vivo* blood–CO_2 dissociation curve will be somewhat larger than the Bunsen solubility coefficient for CO_2, because during the short stay of the blood in the capillaries some bicarbonate will be formed at an uncatalysed rate, and also some carbamino–Hb.

The present data clearly show that infusion of benzolamide, in a dose sufficiently large to inhibit the enzyme in red cells, resulted in an appreciable (56%) reduction in the slope of the ventilatory CO_2 response curve and a considerable decrease in the mean value of B of 4·1 kPa. The cause of this effect is most probably to be found in a decrease in slope of the blood–CO_2 dissociation curve during inhibition of erythrocyte carbonic anhydrase. We explain this as follows. In a carotid body denervated cat we consider the P_{CO_2} of brain tissue (P_{t,CO_2}) to be the stimulus for the central chemoreceptors. Because the CO_2 response curve relates minute ventilation to the P_{CO_2} in arterial blood (P_{a,CO_2}), we have to express P_{t,CO_2} as a function of P_{a,CO_2}. To accomplish this, we use a steady-state mass balance equation for CO_2 of a brain compartment as originally proposed by Read & Leigh (1967) and modified by Berkenbosch, Bovill, Dahan, DeGoede & Olievier (1989) (see eqn (A2) of the Appendix). This equation shows that besides P_{a,CO_2}, P_{t,CO_2} depends on brain metabolism and blood flow, and on the slope of the blood–CO_2 dissociation curve. Since brain blood flow is coupled to P_{t,CO_2} (eqn (A3)), it follows that, over the range covered experimentally, P_{a,CO_2} is linearly related to P_{t,CO_2}. The slope and intercept of this relation depend on the slope of the blood–CO_2 dissociation curve and on the parameters describing the relation between brain blood flow and P_{t,CO_2}. Since benzolamide hardly crosses the blood–brain barrier, we assume that brain metabolism and the relation between brain blood flow and P_{t,CO_2} remained the same. It follows then (eqn (A4)) that the slope and intercept of the relation between P_{t,CO_2} and P_{a,CO_2} depend on the slope l of the blood–CO_2 dissociation curve only. In Fig. 6, three lines relating P_{t,CO_2} to P_{a,CO_2} at three different constant values of l are shown. Line A was experimentally found by Olievier, Berkenbosch, VanBeek, DeGoede & Quanjer (1982) in the anaesthetized cat at a normal value l of 0·024 ml ml^{-1} kPa^{-1} (Vis, 1981). Lines B and C are simulated lines at 50 and 25% of the normal values for l, respectively. Line C is a fairly good approximation of the line existing after benzolamide infusion, since we calculated a value for l of 0·0069 ml ml^{-1} kPa^{-1} in this situation (see below).

From Fig. 6 it is obvious that a decrease in l leads to a decrease in slope of the line relating P_{t,CO_2} to $P_{a,CO2}$. We estimate the resulting decrease in the slope of the $\dot{V}_I$–P_{a,CO_2} relationship as follows. In the Appendix we have derived a mathematical expression relating the steady-state slope of the ventilatory CO_2 response curve to the slope of the *in vivo* blood–CO_2 dissociation curve (eqn (A12)). We have done this by using the same steady-state mass balance equation for CO_2 of a brain compartment as mentioned above (see eqn (A2)). From the observed slope ratio of the steady-state CO_2 response curves before and after administration of benzolamide of 2·28 we thus calculated a slope ratio of the *in vivo* blood–CO_2 dissociation curves after and before drug infusion of 0·288. Considering that the CO_2 dissociation curve of normal cat's blood with a

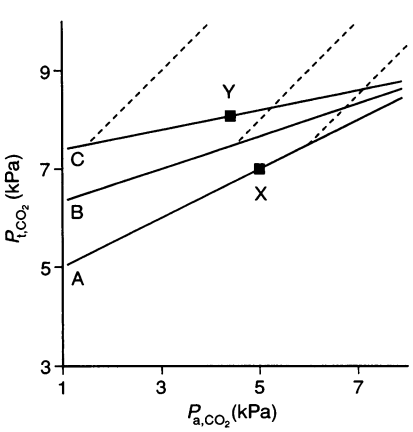

Figure 6. Steady-state relation between P_{t,CO_2} and P_{a,CO_2}
Steady-state relation between P_{t,CO_2} and P_{a,CO_2} at different values of the *in vivo* slope l of the blood–CO_2 dissociation curve. The values for l were 0·024, 0·012 and 0·006 ml ml^{-1} kPa^{-1} for lines A, B and C, respectively. The dashed lines with slopes equal to unity are drawn starting from a P_{t,CO_2} of 7·5 kPa and represent the relations, at the three different values of l, between P_{t,CO_2} and P_{a,CO_2} if brain blood flow would not respond to changes in P_{t,CO_2}. It can be seen that the lower the value of l, the larger the contribution of a vasomotor response to the extent to which a given change in P_{a,CO_2} is reflected in the brain: the smaller the value of l, the larger the difference in slopes between the continuous and dashed lines. Points X and Y refer to the conditions before and after benzolamide administration, respectively.

$P_{CO_2} > 2$ kPa has a slope of $2{\cdot}4 \times 10^{-2}$ ml ml^{-1} kPa^{-1} (Vis, 1981), we calculate that after benzolamide it decreased to $6{\cdot}9 \times 10^{-3}$ ml ml^{-1} kPa^{-1}. This value is somewhat larger than the Bunsen solubility coefficient for CO_2 of $5{\cdot}1 \times 10^{-3}$ ml ml^{-1} kPa^{-1}, as it should be. A similar approach was applied by Adams & Johnson (1990) to calculate the influence of changes in brain blood flow and/or in the slope of the blood–CO_2 dissociation curve on the difference between brain tissue and arterial P_{CO_2} after intravenous infusion of acetazolamide. The decrease in intercept B of the CO_2 response curve after benzolamide infusion was estimated at 6·0 kPa (see Appendix). In this study we found a decrease of 4·1 kPa. Note, however, that after benzolamide infusion the arterial P_{CO_2} of the blood entering the brainstem was 0·6 kPa lower than the equilibrium P_{a,CO_2}. Consequently, the shift in the intercept of the *in vivo* CO_2 response curve in this study was 4·7 kPa.

It was inferred earlier (see Results) that during inhibition of carbonic anhydrase the arterial P_{CO_2} of the blood entering the brainstem is about 0·6 kPa lower than the steady-state equilibrium arterial P_{CO_2}. So when benzolamide is infused at a constant equilibrium P_{a,CO_2} of 5 kPa, the blood will enter the brainstem with an arterial P_{CO_2} of about 4·4 kPa. However, due to the decrease in l, P_{t,CO_2} will rise from 6·9 to about 8 kPa (cf. points X and Y in Fig. 6). Following the relation between brain blood flow and P_{t,CO_2} (cf. eqn (A4)), this will result in a rise in cerebral blood flow of about 100%. It thus appears that if we assume that with inhibition of carbonic anhydrase brain blood flow rises via an effect on P_{t,CO_2} only (cf. Vorstrup, Henriksen & Paulson, 1984; Ringelstein, VanEyk & Mertens, 1992), we are able to estimate a change in brain blood flow which corresponds well with reported increases in cerebral blood flow by acetazolamide in man, dog and rat (Severinghaus & Cotev, 1968; Vorstrup *et al.* 1984; Frankel, Garcia, Malik, Weiss & Weiss, 1992).

Inhibition of central nervous system carbonic anhydrase

We administered methazolamide intravenously because, in this way, we wished to reach all carbonic anhydrase-containing neurones and glial cells directly or indirectly participating in the control of breathing. If, in the central nervous system, carbonic anhydrase is located primarily in first order sensory neurones (see Neubauer, 1991 for references), the enzyme could be a useful tool in the identification of chemoreceptors and in the elucidation of their mechanism of action. The enzyme is localized in neurones within the classical chemosensitive areas in the rostroventrolateral medulla (Ridderstråle & Hanson, 1985) and in neurones from cell cultures of dorsal medulla and hypothalamus (Neubauer, 1991). This possibly widespread distribution of carbonic anhydrase in neurones might be consistent with the idea of a more dispersed location of central chemoreceptors rather than that of a unique location in the rostroventrolateral medulla (Dean, Lawing & Millhorn, 1989; Coates, Li & Nattie, 1993).

If central chemoreceptors do indeed have a widespread distribution, topical application of carbonic anhydrase inhibitors on the ventral medullary surface as used by Andreatta van Leyen, Averill & Guertzenstein (1990) and Coates, Li & Nattie (1991) would not affect all chemoreceptors. A limitation of topical administration is that it might cause local changes in pH and other effects unrelated to inhibition of carbonic anhydrase. It is difficult therefore to compare the results of local application with those obtained in the present study. Another approach was used by Adams & Johnson (1990) who perfused the ventrolateral surface of spontaneously breathing rats with cerebrospinal fluid to which acetazolamide was added. The authors found that their largest dose of acetazolamide increased the ventilatory response to CO_2-rebreathing by 40%. The fact that we, by using a technique by which presumably all chemoreceptors were reached, found a similar (see Table 1) increase in CO_2 responsiveness, could then be interpreted to mean that the cells responsible for this increased sensitivity are located in superficial ventral medullary structures. More studies are needed to investigate this.

From Fig. 5 it is evident that methazolamide has an effect on ventilation that was opposite to that of benzolamide. The administered dose of 20 mg kg^{-1} was high enough to completely block CNS carbonic anhydrase (Maren, 1977). We assume that methazolamide, if infused in a condition in which the erythrocyte enzyme is already inhibited, does not change brain metabolism, the relationship between $\dot{Q}$ (the brain blood flow density) and P_{t,CO_2}, the slope of the blood–CO_2 dissociation curve and the parameters h and γ. It can then be seen (eqn (A10)) that an explanation for the increase in x-intercept of the response curve can be found in an increase in offset B_t, possibly caused by an altered neuronal excitability. In this context it is worthwhile to mention that sulphonamides exert their anticonvulsant effect probably by reducing brain excitability via inhibition of glial carbonic anhydrase (Woodbury & Kemp, 1982; Woodbury, Engstrom, White, Chen, Kemp & Chow, 1984). It should also be mentioned here that inhibition of carbonic anhydrase within the peripheral chemoreceptors decreases the carotid body output *in vitro* and probably also *in situ* (see Itturiaga, 1993 for references). The increase in slope of the CO_2 response curve could be explained by a decreased brain intracellular buffering of H^+ ions to CO_2 by inhibition of carbonic anhydrase as demonstrated by Kjällquist, Messeter & Siesjö (1970). With a given change in tissue P_{CO_2}, an intracellular pH sensor would then be stimulated more strongly, giving rise to a greater ventilatory response.

On the basis of the present results it is to be expected that inhibiting the enzyme in all tissues simultaneously will result in a ventilatory effect in which the effect of erythrocyte inhibition dominates. This view is strengthened by additional data in three cats which were given

20 mg kg^{-1} methazolamide without prior administration of benzolamide. The mean decrease in the ventilatory CO_2 response slope was only 16% in these animals, and the mean decrease in intercept only 1·9 kPa.

In clinical practice, inhibitors of carbonic anhydrase, almost invariably acetazolamide, are commonly given orally in quantities of about 2·5 mg kg^{-1}, too small to inhibit effectively the erythrocyte enzyme. It remains to be investigated whether this dose is sufficient to block central neuronal carbonic anhydrase completely.

APPENDIX

The mass balance equation for CO_2 of a brain compartment can be written as (Read & Leigh, 1967; Berkenbosch *et al.* 1989):

$$\frac{dP_{t,CO_2}}{dt} = \frac{l}{l_t}\dot{Q}(P^c_{a,CO_2} - P_{t,CO_2}) + \frac{(1-\gamma)(\dot{M}-h)}{l_t}, \quad (A1)$$

where P^c_{a,CO_2} and P_{t,CO_2} denote the arterial P_{CO_2} of the blood entering the brainstem, and brain tissue P_{CO_2}, respectively; $\dot{Q}$ and $\dot{M}$ are the brain blood flow density and brain metabolism density; l and l_t, the slopes of the linearized blood and brain tissue–CO_2 dissociation curves; h, the Haldane parameter and γ, a parameter which locates P_{t,CO_2} between P^c_{a,CO_2} and the cerebral venous P_{CO_2} (P_{v,CO_2}). In the steady-state eqn (A1) reduces to:

$$P_{t,CO_2} = \frac{(1-\gamma)(\dot{M}-h)}{l\dot{Q}} + P^c_{a,CO_2}. \quad (A2)$$

The cerebral blood flow is assumed to be coupled to P_{t,CO_2} in a hyperbolic fashion, with the shape factor a and the asymptote b (cf. Dahan, Berkenbosch, DeGoede, Olievier & Bovill, 1990):

$$\dot{Q} = \frac{a}{(b - P_{t,CO_2})}. \quad (A3)$$

Substituting eqn (A3) in eqn (A2) yields a linear relation between P_{t,CO_2} and P_{a,CO_2}:

$$P_{t,CO_2} = \frac{1}{1+\frac{(1-\gamma)(\dot{M}-h)}{al}} P_{a,CO_2} + \frac{b}{1+\frac{al}{(1-\gamma)(\dot{M}-h)}}. \quad (A4)$$

A linear relation between P_{t,CO_2} and P_{a,CO_2}, with slope α and intercept β, was indeed found experimentally (Pontén & Siesjö, 1966) according to:

$$P_{t,CO_2} = \alpha P^c_{a,CO_2} + \beta. \quad (A5)$$

For $P_{t,CO_2} = b$ (at infinite high blood flow), P_{t,CO_2} and P^c_{a,CO_2} are equal so that using eqns (A3) and (A5) it follows that:

$$b = \frac{\beta}{1-\alpha}. \quad (A6)$$

We assume that in peripheral chemoreceptor denervated animals ventilation ($\dot{V}_I$) is linearly related to P_{t,CO_2} so that:

$$\dot{V}_I = S_t(P_{t,CO_2} - B_t), \quad (A7)$$

in which S_t is the CO_2 sensitivity at the site of the central chemoreceptors and B_t an offset. Ventilation as function of the P^c_{a,CO_2} is

$$\dot{V}_I = S^c(P^c_{a,CO_2} - B^c), \quad (A8)$$

with the slope S^c and the x-intercept B^c.

From eqns (A2), (A5) and (A7) it follows that the slope S^c is:

$$S^c = \alpha S_t = \frac{1}{1+\frac{(1-\gamma)(\dot{M}-h)}{al}} S_t. \quad (A9)$$

Using eqns (A5), (A6), (A7), (A8) and (A9) the x-intercept can be written as:

$$B^c = \frac{B_t - b}{\alpha} + b. \quad (A10)$$

From eqn (A9) it follows that:

$$l = \frac{\alpha(1-\gamma)(\dot{M}-h)}{a(1-\alpha)}. \quad (A11)$$

Since benzolamide does not cross the blood–brain barrier we assume that the parameters γ, a, b, h and S_t remained constant after administration of the drug. Consequently, we attribute the change in S^c by benzolamide entirely to a change in l, i.e. to a change in the CO_2 dissociation curve of blood. Introducing the subscript $_b$ for the parameters after benzolamide administration the ratio l_b/l can be written as

$$\frac{l_b}{l} = \frac{1-\alpha}{\frac{S^c}{S^c_b}\alpha}, \quad (A12)$$

where we have used the relation

$$\alpha_b = S^c_b \frac{\alpha}{S^c}. \quad (A13)$$

The P_{iv,CO_2} represents, to a good approximation, the arterial P_{CO_2} of the blood entering the brainstem. Furthermore it was found that after benzolamide administration:

$$P_{iv,CO_2} = 0{\cdot}97\ P_{a,CO_2} - 0{\cdot}6. \quad (A14)$$

To establish a connection with the experimentally determined CO_2 sensitivity and intercept we note that before administration of benzolamide S^c and B^c are equal to the experimentally found values of S and B (see data analysis). After administration of benzolamide S^c_b is equal to the observed sensitivity S_b, while the experimentally found value B_b is equal to $B^c_b + 0{\cdot}6$ (see eqn (A14)).

We found for the ratio S/S_b a value of 2·28. A value of 0·48 for α and 4·45 for β (see eqn (A5)) was reported by Olievier *et al.* (1982) taking P_{t,CO_2} equal to CSF P_{CO_2} (Pontén &

Siesjö, 1966). It thus follows that $\alpha_b = 0{\cdot}21$. We estimate that when the decrease in slope of the ventilatory response to changes in P_{a,CO_2} is entirely due to the decrease in l, the ratio l_b/l is 0·288. We calculate using eqn (A10) that the intercept $B^c (= B)$ decreased from 3·89 kPa during control (Table 1) to −2·10 kPa after benzolamide administration. This decrease in B, from B^c to B_b^c, of 6·0 kPa should be compared with a measured shift of (4·1 + 0·6) = 4·7 kPa.

When benzolamide is infused at a constant equilibrium P_{a,CO_2} of about 5 kPa (as in the present study), the arterial blood will enter the brainstem with a P_{CO_2} of about 4·4 kPa. Using eqns (A5) and (A6) it can be estimated that P_{t,CO_2} will increase from 6·9 to 7·7 kPa. Assuming that parameters a and b (eqn (A3)) remained constant, this results in an estimated increase in cerebral blood flow of 100%.

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