ANALYSIS OF H_2 CLEARANCE CURVES USED TO MEASURE BLOOD FLOW IN RAT SCIATIC NERVE

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SUMMARY

1. By use of the H_2 clearance technique, blood flow was measured in the sciatic nerve of healthy, anaesthetized Sprague–Dawley rats at rest, during inferior vena cava occlusion and during 5-hydroxytryptamine infusion. The purpose was to clarify the mechanisms underlying the biexponential curves which are commonly obtained using this technique.

2. An analysis of the frequency distribution of rate constants of 270 nerve and thirty-three arterial samples indicated that H_2 clearance rates cluster below 20 ml min^{-1} 100 g^{-1} and between 70 and 100 ml min^{-1} 100 g^{-1}. This suggests that at least two compartments are present.

3. The contribution of diffusion was studied by recording H_2 clearance immediately following circulatory arrest. Slow clearance rates (median = 2·4 ml min^{-1} 100 g^{-1}) were observed, indicating that diffusion is not likely to contribute significantly to nutritive flow under most situations.

4. The contribution of arteriovenous shunts to H_2 clearance was assessed by determining H_2 clearance during inferior vena cava occlusion and the infusion of 5-hydroxytryptamine. Both manoeuvres caused abolition of, or a significant reduction in the weight of, the fast component which indicates that this compartment is closely related to arteriovenous shunts in nerve.

5. By use of a multi-compartmental model, it was shown that H_2 clearance should follow a multi-exponential course, where the weights of the components reflect the relative volumes of each compartment and the exponents represent the relative flow (i.e. flow per unit volume) in each compartment.

6. By use of other mathematical models, estimates were made for the clearance rates attributable to polarographic oxidation of H_2 at the tip of the microelectrode (0·2 ml min^{-1} 100 g^{-1}) and to diffusion to air (2 ml min^{-1} 100 g^{-1}). The latter estimate is very close to the measured value of 2·4 ml min^{-1} 100 g^{-1}.

7. These findings indicate that it is possible to separately assess nutritive and non-nutritive flow by application of biexponential analysis to H_2 clearance curves. The data suggest that the fast component of a H_2 clearance curve is closely associated with arteriovenous shunts, while the slower component is likely to represent capillary flow. Processes such as diffusion to air or oxidation of H_2 by the electrode are very slow and therefore are unlikely to distort the assessment of blood flow by using this technique.

INTRODUCTION

Biexponential curves are frequently obtained using H_2 clearance techniques to measure blood flow in brain (Fieschi, Bozzao, Agnoli, Nardini & Bartolini, 1969; Pasztor, Symon, Dorsch & Branston, 1973), kidney, muscle (Aukland, Bower & Berliner, 1964; Aukland, 1965) and peripheral nerve (Low & Tuck, 1984; McManus & Low, 1988). In brain, the two components are thought to represent blood flow in the grey and white matter. Two such discrete anatomical compartments are not present, however, in skeletal muscle or peripheral nerve. The slower component in muscle and peripheral nerve correlates well with nutritive blood flow in muscle (Aukland, Akre & Leraand, 1969) and nerve (Low & Tuck, 1984), but the mechanism(s) of the fast component are poorly understood. Veno-arterial counter-current shunting of H_2 has been proposed as an explanation for the fast component in skeletal muscle (Aukland *et al.* 1969), but no previous studies have addressed the question in peripheral nerve.

The separation of non-nutritive from nutritive blood flow is an important consideration in many tissues. Radiolabelled microspheres are usually used; for instance, 15 and 175 μm diameter spheres can be injected simultaneously to determine total and capillary blood flow, respectively (Hales, 1981). However, microsphere technique requires the use of isotopes and killing the animal. We hypothesized that it might be possible to separate the two components of blood flow by relating these to the different components of the H_2 clearance curve.

There are several mechanisms by which H_2 may be cleared from tissues. These are capillary blood flow, diffusion into larger vessels such as arterioles or arteriovenous (AV) shunts, diffusion into adjacent tissues which are cleared at a faster rate ('concentration disequilibrium'), diffusion to air (where measurements are made in exposed preparations) and consumption of H_2 by the polarographic oxidation reaction. In this series of experiments, we have attempted to evaluate and apportion the individual contributions of the above clearance mechanisms to the overall H_2 clearance. The studies suggest that the fast component exists mainly because of flow in AV shunts and that there is an ultra-slow component attributable to diffusion. Such dissection of the H_2 clearance curve permits the separate determination of nutritive and non-nutritive flow in peripheral nerve.

METHODS

Nerve blood flow recordings

Preparatory procedures have been previously described (Low & Tuck, 1984) and will be summarized here. Male Sprague–Dawley rats, weighing 230–300 g, were anaesthetized with Inactin (5-ethyl-(1-methyl-propyl)-2-thiobarbituric acid; Byk Gulden, Konstanz; 100 mg kg^{-1}, I.P.) which induces general anaesthesia in rats for 4–6 h. The animals were paralyzed with tubocurarine HCl (15–20 U kg^{-1} intra-arterially or intraperitoneally) which maintained muscle relaxation for 3–4 h. Depth of anaesthesia was assessed by continuous monitoring of systemic arterial blood pressure, which showed no spontaenous fluctuations nor changes in response to manipulation of tissues under sufficiently deep anaesthesia. When such fluctuations appeared, usually towards the end of an experiment, additional increments of 8–12 mg kg^{-1} Inactin were given by slow I.A. injection. When necessary, additional doses of I.A. 3–5 U kg^{-1} tubocurarine were given slowly to abolish muscle twitches. Ventilation was maintained, via a tracheostomy, with a rodent ventilator (Harvard Apparatus, Millis, MA, USA). The left common carotid artery (CCA) was cannulated to permit continuous pressure monitoring and to allow arterial blood sampling for estimation of pH, P_{CO_2}

and P_{O_2}. Rectal temperature was monitored and controlled by a heating lamp linked to a feedback-control unit.

The left sciatic nerve was exposed by dissection through the gluteal muscles and a well was created in the muscle. Epineurial and perineurial tissues were thinned out by sharp dissection under an operating microscope ($40 \times$) taking care to avoid disruption of blood vessels. The well was filled with mineral oil maintained at $33.5\ °C$ with a temperature probe and heating lamp. Hydrogen-sensitive microelectrodes were constructed from glass tubing, copper and platinum wire as previously described (Low & Tuck, 1984). The tip of the electrodes measured $2-5\ \mu m$ in diameter. Only electrodes with a linear response to H_2 *in vitro* were used. The electrode was advanced into the endoneurium using a micro-manipulator and polarized at $+250\ mV$ by a current-sensitive amplifier (Microsensor Diamond, Electro-Tech Inc., Ann Arbor, MI, USA). A length of agar–KCl filled polyethylene tubing, sutured in the subcutaneous tissues, was used as the reference electrode. This was inserted into a flask containing $2\ M$-KCl and an Ag–AgCl electrode connected to the microammeter.

The animal was given $10\%\ H_2$ to breathe by adding H_2 to the inlet port of the ventilator and adjusting the concentrations of N_2 and O_2 to maintain a constant fraction of inspired oxygen. The current generated at the electrode tip was monitored until a stable level was reached. The H_2 was abruptly turned off and the entire clearance curve was recorded on a Grass chart recorder (Grass Instruments, Quincy, MA, USA). The first minute of the recording was discarded and the subsequent 10 min analysed by entering the data points into a Hewlett–Packard computer (HP9845T). Non-linear regression analysis was performed to resolve the curves into mono- or biexponential functions using the method of least-squares, with a convergence coefficient of 0·005 (Hewlett–Packard non-linear regression program based on the Marquardt algorithm).

Diffusion experiments

In nerve blood flow (NBF) measurements, the sciatic nerve was surrounded by a pool of mineral oil exposed to the air. In order to assess the contribution of diffusion to H_2 clearance, a series of post-mortem clearance curves was recorded. Since there was no blood flow, the reduction in H_2 current with time would be due to diffusion alone. These measurements were made after the completion of other experiments using H_2 polarography. The nerve was saturated with H_2 by allowing the animal to breathe H_2 via the ventilator until a stable current response was obtained. The animal was killed by rapid injection of concentrated KCl into the left common carotid artery causing cardiac arrest. The decline in the H_2 current was followed over the next 20–30 minutes and analysed using the non-linear regression program described above.

Inferior vena cava occulsion experiments

To assess the contribution of arteriovenous (AV) shunts to H_2 clearance in nerve, we made use of the known propensity for AV shunts to close in response to increases in venous pressure (Sherman, 1963). We temporarily occluded the inferior vena cava (IVC) by tightening a noose made of PE10 tubing, as described for the aortic noose (Low, Nukada, Schmelzer, Tuck & Dyck, 1985). A reduction in the number of patent shunts or total shunt flow would be expected to reduce the weight of the component contributed by the shunt. Three or four sequential measurements were made in four rats with the noose open, closed, opened again and closed again. Three minutes were allowed to elapse between each manoeuvre to allow blood pressure and other circulatory changes to stabilize after each such manoeuvre. In contrast to the other measurements, the wash-outs were analysed from the onset of the clearance process, to ensure accurate determination of the relative weights of the components. The curves were resolved into biexponential functions. In addition, the weight of each component was determined, and the weighted mean flow calculated using the following formulae:

$$W_1 = \frac{a}{a+b}\ 100\%$$

$$W_2 = 100\% - W_1$$

$$F_1 = k_1 \lambda;\ F_2 = k_2 \lambda$$

$$F_w = W_1 F_1 + W_2 F_2$$

where the clearance function is described by $f(t) = a \exp(-k_1 t) + b \exp(-k_2 t)$, where a and b are

the contribution of the fast and the slow exponential (respectively) to the wash-out curve. W_1 is the weight of the faster component and F_w is the weighted mean flow. The clearance rate constants (k_1, k_2) were converted to blood flow values (units = ml min^{-1} 100 g^{-1}) by use of the tissue–blood partition coefficient (λ). Hydrogen is extremely soluble in body tissues, is highly diffusible, and has a tissue–blood partition coefficient close to unity $(\lambda = 100$ ml 100 g^{-1}) for a variety of tissues (Aukland, Bower & Berliner, 1964).

In contrast to the previous report from this laboratory (Low & Tuck, 1984), we used the expression $(W_1 = 100\, a/[a+b])$ to calculate the weight of the fast component, and from this to calculate the weighted mean flow. This expression is appropriate since we allowed the tissues to become saturated with hydrogen over several minutes, approximating steady-state conditions. The previously quoted equation $(W_1 = 100\, a\, k_2/[a\, k_2+b\, k_1]$ is only applicable when a bolus injection of tracer is made and correction for non-steady-state starting conditions is required (Hoedt-Rasmussen, 1967). This difference in the calculations is the explanation for the different values for W_1 and F_w recorded in this report, compared with the previous paper.

5-Hydroxytryptamine (5-HT) infusions

In a further attempt to address the question of AV shunts, a series of experiments was performed in which 5-HT (Sigma Chemicals, St Louis, MO, USA), which has been shown to reduce flow in arteriovenous anastomoses, was infused in the right jugular vein (Saxena & Verdouw, 1982, 1985). Preliminary studies in which skin blood flow in the ear was monitored using a Laser Doppler Velocimeter (LDV; LD5000, Medpacific, Seattle, WA, USA), and arterial blood pressure (BP) was measured, were used to define the dose–response characteristics in our animals. A dosage of 5–10 μg 5-HT kg^{-1} min^{-1} was used (as the base) in the subsequent studies.

Fifteen rats were prepared as described above. Following a baseline measurement, infusions of 5-HT via a cannula in the right jugular vein were given at rates of 5, 10 and, rarely, 20 μg kg^{-1} min^{-1}. After a vasoactive effect was registered by a change in the LDV signal or the BP, a H_2 clearance curve was recorded. The infusion was continued for the first 10 min of this curve which was analysed from the onset of the wash-out so that weights could be accurately assigned. After completion of this 5-HT infusion curve, a further H_2 wash-out was performed to assess the degree of recovery from the drug effect. Where feasible, two or more cycles of 5-HT infusion and recovery were recorded.

Data were excluded from the analysis if the infusion caused a significant fall in the BP (either < 110 Torr absolute or a reduction of > 30 Torr) or if no evidence of a vasoactive effect was seen in the LDV or BP recordings. Where no recovery was evident (in three animals), the initial baseline–treatment data was still included in the analysis. For each 'effective perturbation', defined by evidence of a vasoactive effect in the LDV or BP, 'baseline' was defined as the most recent clearance curve prior to that 'perturbation'. Only 'effective' perturbations were included in the 5-HT infusion group. Where repeated recovery curves were run, only the last one was used in the analysis.

'Baseline' values were compared with '5-HT infusion' values using a two-tailed, grouped t test for the following parameters: fast flow rate (F_1); slow flow rate (F_2); weight of the fast component (W_1); and the weighted mean flow (F_w). Likewise, the '5-HT infusion' data were compared with the 'recovery' data.

Arterial recordings

To assess the effect of rapid clearance of H_2 from the arterial system, H_2 clearances were recorded from the arterial wall of the proximal and distal common carotid artery (CCA), abdominal aorta, femoral artery, popliteal artery and a nutrient arteriole to the sciatic nerve (confirmed as such by rapid opacification after intra-arterial injection of Evans Blue dye). Although rapid clearance is expected (and has been described) using intravascular electrode recording, we used the alternative and simpler approach of arterial wall microelectrode placement to study arterial clearance rates. For simplicity, these curves will be referred to as arterial curves. In addition, clearances were recorded using the femoral vein and IVC. In each case, satisfactory wash-outs were recorded by placing the tip of the microelectrode in the vessel wall adventitia which was then covered with mineral oil to prevent it from drying out. For these recordings, which often had a very fast initial component, high frequency filter settings of 3 or 15 Hz were used to avoid blunting of the fast component. As for the IVC experiments, data were analysed from the earliest part of the wash-out curve, but the experiment was continued for only 2–3 min as the current had fallen to < 10 % of initial values by this time.

Analysis of all H₂ clearance curves

All polarographic measurements obtained by one investigator (T.J.D.) over the previous 6 months were reviewed and a frequency distribution of rate constants plotted. These included baseline data and experimental data after nerve ischaemia, as well as NBF measurement after IVC occlusion and infusion of 5-HT. Arterial adventitial recordings, described below, were included in a second analysis.
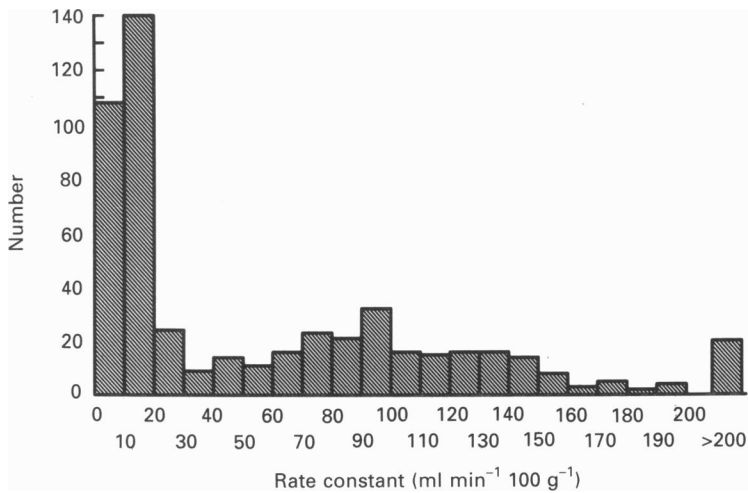

Fig. 1. Frequency distribution of rate constants – nerve blood flow data. Frequency distribution of all rate constants derived from H₂ polarographic nerve blood flow measurements obtained by one investigator (T.J.D.).

Statistical methods

Data are listed as group mean ± standard error of the mean (S.E.M.) unless otherwise stated. Groups were compared using Student's *t* test, with the Dunnett correction for multiple comparisons (Dunnett, 1955). Although paired analysis was possible in many of the experiments, this did not change the conclusions and, generally, had lower statistical power.

RESULTS

Analysis of H₂ clearance curves

Of 270 H₂ clearance curves recorded from nerve, only twenty-four were purely monoexponential. In addition, there were thirty-three arterial curves, all of which fitted biexponential functions. The frequency distributions of the nerve data and all data combined are shown in Figs 1 and 2. There are definite peaks in three discrete regions: < 20, 70–100 and 800–1000 ml min⁻¹ 100 g⁻¹. In addition, there may be a smaller clustering at 120–140 ml min⁻¹ 100 g⁻¹.

Contribution of diffusion

In all animals studied, an initial faster component was replaced with a slow clearance of H₂ at rates ranging from 1·1–11·9 ml min⁻¹ 100 g⁻¹. The distribution is depicted in Fig. 3. The mean clearance was 3·04 ± 2·4 ml min⁻¹ 100 g⁻¹ with a median
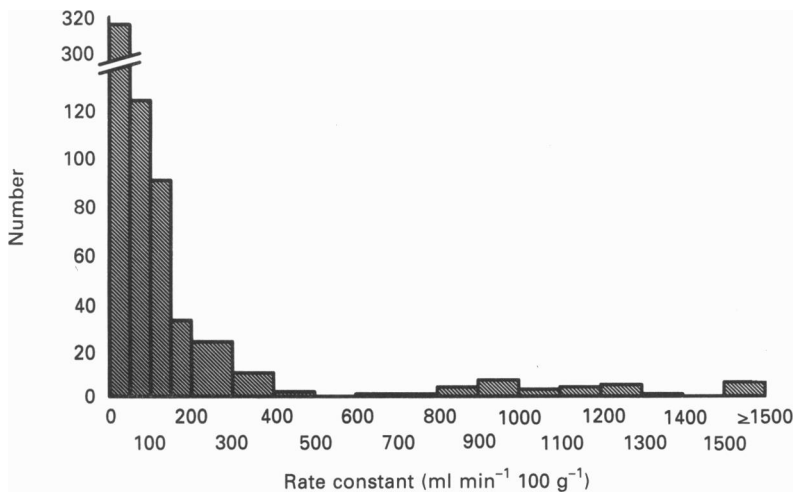
Fig. 2. Frequency distribution of rate constants – all data. Frequency distribution of all rate constants derived from nerve blood flow measurements and arterial H_2 clearance curves determined by one investigator (T. J. D.)
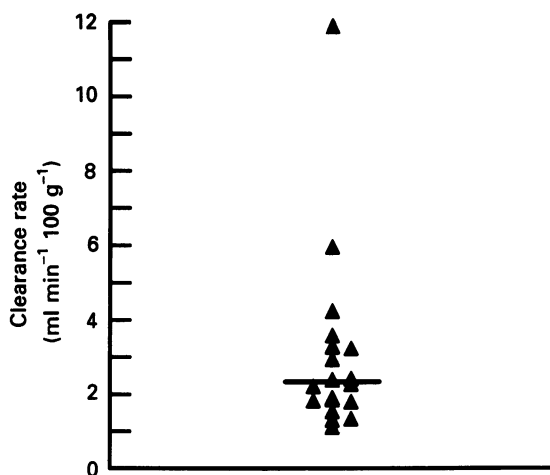

Fig. 3. H_2 clearance rates – diffusion. Scatter diagram showing the distribution of H_2 clearance rates attributable solely to diffusion to air. The bar represents the median clearance rate of 2·4 ml min^{-1} 100 g^{-1}.

of 2·37 ml min^{-1} 100 g^{-1}. If the observation of 11·9 is excluded (since it lies more than three standard deviations from the mean), the distribution becomes less skewed with a mean of 2·57 ± 1·2 ml min^{-1} 100 g^{-1}, a median of 2·29 ml min^{-1} 100 g^{-1} and the 95th percentile = 4·25 ml min^{-1} 100 g^{-1}. In reviewing the original records, it was noted that the preparation with the outlying value had required frequent addition of oil to the bath and may have had only a thin layer covering the nerve, giving rise to a spuriously fast wash-out. In a recent study (Takeuchi & Low, 1987), we regressed H_2 polarographically derived NBF against that determined with laser Doppler

TABLE 1. The effect of inferior vena cava (IVC) occlusion on H_2 clearance rates

	F_1 ml min^{-1} 100 g^{-1}	F_2 ml min^{-1} 100 g^{-1}	W_1 percentage	F_w ml min^{-1} 100 g^{-1}
Noose off $n = 8$	111 ± 13	11.7 ± 1.1	55.1 ± 5.7	64.0 ± 7.6
Noose on $n = 6$	116 ± 15	7.7 ± 0.6	15.7 ± 2.3	24.3 ± 2.7
t statistic	0.236	3.196	6.458	4.943
P value	n.s.	< 0.01	< 0.001	< 0.001

'Noose off' indicates measurements made before or after IVC occlusion, while 'Noose on' indicates measurements made during the period of IVC occlusion. Data are expressed as mean $\pm$ s.e.m. F_1 = fast rate constant $\times \lambda$; F_2 = slow rate constant $\times \lambda$; W_1 = weight of the fast component and F_w = weighted mean flow.
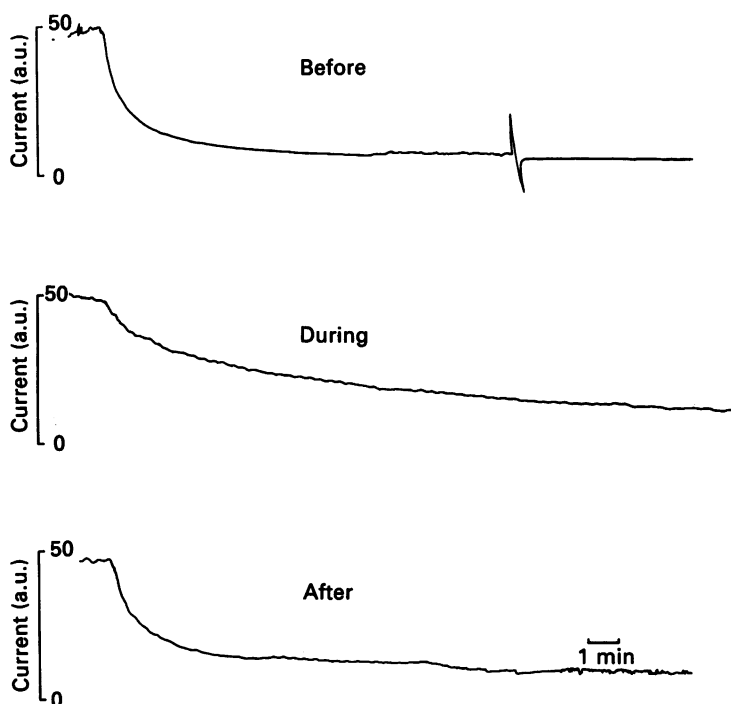

Fig. 4. Effect of inferior vena cava (IVC) occlusion on H_2 clearance curves. Typical examples of H_2 clearance curves recorded consecutively before, during and after IVC occlusion. The weights of the fast components were 76, 26 and 61 %, respectively. The BP remained between 105 and 110 Torr during these measurements. a.u. = arbitrary units.

technique and found an intercept at 2·6 ml min^{-1} 100 g^{-1}, presumably reflecting the contribution of diffusion.

It is possible to compare these empirical values with the clearance rate predicted from theoretical analysis. Mathematical modelling of diffusion yielded a rate constant of 0·019 min^{-1}, equivalent to a flow of 1·9 ml min^{-1} 100 g^{-1} (see Mathematical Modelling in Appendix).

*Contribution of arteriovenous (AV) shunts: inferior vena cava (IVC) occlusion
experiment*

Eight rats were studied using IVC occlusion to close AV shunts. No significant
change in the BP resulted from this manoeuvre. Two complete cycles of occlusion
and recovery were achieved in two animals, while in the others only one cycle was
recorded. Table 1 lists the rate constants (converted to flows in ml min^{-1} 100 g^{-1}),
relative weights and weighted mean flow for the different conditions employed in this
experiment. There was a statistically significant reduction in the slower component
($P < 0.001$) and the weighted mean flow ($P < 0.001$). A typical series of wash-out
curves is shown in Fig. 4. A striking reduction in the contribution of the faster
component ($P < 0.001$; Table 1) upon IVC occlusion is evident. This effect was
reversible on release of the IVC noose and reproducible on repeating these
manoeuvres.

These data suggest that the fast component (F_1) is closely related to high flow
vessels which are liable to close in response to increases in venous pressure (i.e. AV
shunts). The reduction in the slower component (F_2) is consistent with the concept
that this reflects capillary flow, since IVC occlusion would also be expected to reduce
capillary perfusion.

*Contribution of arteriovenous shunts: 5-hydroxytryptamine (5-HT) infusion
experiments*

Of fifteen rats, three were excluded due to unacceptable hypotension during the
infusion or throughout the experiment. In the remaining animals, 5-HT produced
changes in the LDV signal and H$_2$ clearance curve without significant changes in BP
(Fig. 5).

Inspection of the data obtained from the remaining twelve rats showed that there
were two main groups: group A showed a significant reduction in F_2, W_1 and F_w, but
little or no reduction in F_1 (Table 2A), the same pattern of response as that resulting
from IVC occlusion. A typical sequence of clearance curves is shown in Fig. 6. Four
animals showed repeated reproducible recovery to near baseline values, but in three
the drug effects did not resolve in under 2 h and the experiments were terminated.

All flow parameters (F_1, F_2 and F_w) were reduced to less than half during 5-HT
infusion in group B (Table 2B), although demonstrable recovery was present in all.
These reductions were significantly different from baseline values ($P < 0.001$). In
these animals, the LDV effect was usually quite marked, frequently reduced to zero
flow levels, and recovery was often delayed for 1–2 h, factors which would imply a
more vigorous vasoactive effect had occurred. In such cases (group B), flow rates in
all compartments including nutritive flow would be expected to be lower than in
group A. The reduction in F_1, F_2 and F_w (Table 2B) are compatible with this
hypothesis. The global reduction in flows probably accounts for the less striking, but
still highly significant, reduction in W_1 in this group.

Arterial clearance studies

Arterial recordings from four rats yielded biexponential curves with a very fast
clearance ranging from 700 to 1600 ml min^{-1} 100 g^{-1} and a slower clearance of
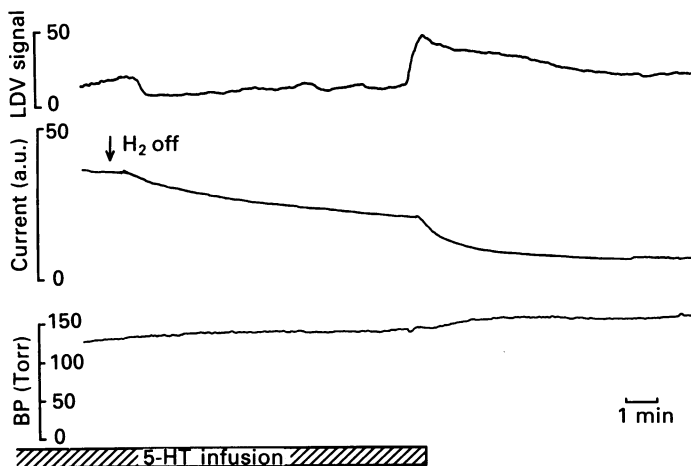
Fig. 5. Effect of 5-hydroxytryptamine (5-HT) on laser Doppler, H_2 clearance and BP. Sample of an experimental record showing the LDV signal (upper trace – arbitrary units), H_2 current (centre trace – arbitrary units) and mean arterial blood pressure (lower trace – arbitrary units). The infusion of 20 μg 5-HT kg^{-1} min^{-1} is indicated by the hatched bar. Note the stable BP and the immediate return of a fast component to the H_2 clearance curve upon discontinuing the infusion.

TABLE 2A. The effect of 5-hydroxytryptamine (5-HT) infusion on H_2 clearance rates (group A)

	F_1 ml min^{-1} 100 g^{-1}	F_2 ml min^{-1} 100 g^{-1}	W_1 percentage	F_w ml min^{-1} 100 g^{-1}
Baseline $n = 12$	$110{\cdot}6 \pm 16$	$17{\cdot}1 \pm 1{\cdot}3$	$47{\cdot}8 \pm 3{\cdot}1$	$63{\cdot}8 \pm 9{\cdot}9$
5-HT infusion $n = 13$	$158{\cdot}6 \pm 26$	$9{\cdot}2 \pm 1{\cdot}1$	$22{\cdot}6 \pm 6{\cdot}6$	$35{\cdot}5 \pm 8{\cdot}1$
Recovery $n = 10$	$102{\cdot}3 \pm 12$	$17{\cdot}0 \pm 1{\cdot}8$	$44{\cdot}9 \pm 4{\cdot}9$	$55{\cdot}4 \pm 7{\cdot}8$
Baseline *vs.* 5-HT	n.s.	$P < 0{\cdot}0001$	$P < 0{\cdot}005$	$P < 0{\cdot}05$
Recovery *vs.* 5-HT	n.s.	$P < 0{\cdot}001$	$P < 0{\cdot}025$	n.s.

These measurements were made in animals in which infusion of 5-HT did not reduce F_1 by more than 15 %. 'Baseline' measurements were made immediately prior to 5-HT infusion; '5-HT infusion' indicates measurements made during an infusion which caused a perturbation in the laser Doppler (LDV) signal and 'recovery' indicates measurements obtained after termination of infusion. Data are expressed as mean ± S.E.M. F_1 = fast rate constant × λ; F_2 = slow rate constant × λ; W_1 = weight of the fast component and F_w = weighted mean flow.

200 ml min^{-1} 100 g^{-1}. The mean values for the rate constants and W_1 are listed in Table 3. The very fast component was similar when measured at sites ranging from the carotid artery down to the arteriolar vessels. The slower rate constant in the vicinity of a peripheral nerve arteriole averaged 65 ml min^{-1} 100 g^{-1}, which compares favourably with fast components commonly recorded in nerve (approximately 70–100 ml min^{-1} 100 g^{-1}).
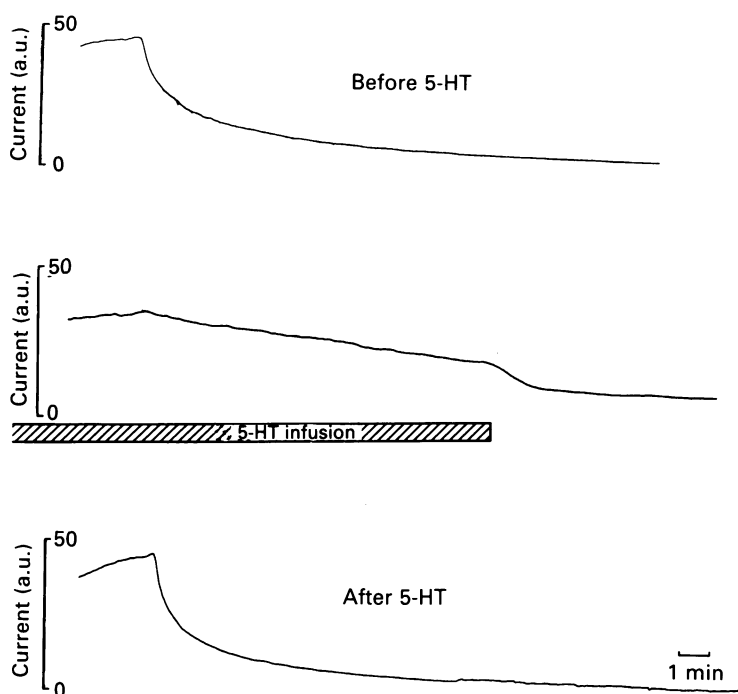
Fig. 6. Effect of 5-hydroxytryptamine (5-HT) infusion on H_2 clearance curves. Alterations in shape of H_2 clearance curves in relation to infusion of 5-HT at $10 \mu g$ kg^{-1} min^{-1} (represented by the hatched bar). Those shown are consecutive H_2 clearance curves before, during and 20 min after the infusion. The weights of the faster components are 52, 0 (monoexponential) and 60%, respectively. The BP was stable at 130 Torr during each recording. The rapid increase in H_2 clearance upon discontinuing the 5-HT infusion is evident in the second tracing. a.u. = arbitrary units.

TABLE 2B. The effect of 5-hydroxytryptamine (5-HT) on H_2 clearance rates (group B)

	F_1 ml min^{-1} 100 g^{-1}	F_2 ml min^{-1} 100 g^{-1}	W_1 percentage	F_w ml min^{-1} 100 g^{-1}
Baseline $n = 10$	$156 \cdot 2 \pm 11$	$15 \cdot 6 \pm 1 \cdot 1$	$46 \cdot 0 \pm 5 \cdot 1$	$79 \cdot 6 \pm 10 \cdot 5$
5-HT infusion $n = 12$	$72 \cdot 3 \pm 7 \cdot 7$	$7 \cdot 6 \pm 1 \cdot 5$	$29 \cdot 0 \pm 4 \cdot 7$	$26 \cdot 6 \pm 25 \cdot 2$
Recovery $n = 10$	$140 \cdot 0 \pm 13$	$15 \cdot 7 \pm 1 \cdot 8$	$36 \cdot 3 \pm 3 \cdot 8$	$62 \cdot 2 \pm 8 \cdot 8$
Baseline vs. 5-HT	$P < 0 \cdot 00001$	$P < 0 \cdot 001$	$P < 0 \cdot 025$	$P < 0 \cdot 0005$
Recovery vs. 5-HT	$P < 0 \cdot 001$	$P < 0 \cdot 003$	n.s.	$P < 0 \cdot 005$

These measurements were made in animals in which 5-HT infusion caused slowing of the fast component. 'Baseline' measurements were made immediately prior to 5-HT infusion; '5-HT infusion' indicates measurements made during an infusion which caused a perturbation in the laser Doppler (LDV) signal and 'recovery' indicates measurements obtained after termination of the infusion. Data are expressed as mean±S.E.M. F_1 = fast rate constant × λ; F_2 = slow rate constant × λ; W_1 = weight of the fast component and F_w = weighted mean flow.

Inspection of the curves and comparisons of the half-times showed that H_2 is cleared from the vessel wall within 1 min in most and under 2 min in all cases. The clearance from arterial blood, assuming $F_1 = 1000$ ml min^{-1} 100 g^{-1} (corresponding to a half-time of 4 s), is more than 96% complete within 20 s.

TABLE 3. Blood pressure and H_2 clearance data at different vessel sites

	F_1 ml min^{-1} 100 g^{-1}	F_2 ml min^{-1} 100 g^{-1}	W_1 percentage	n
Common carotid artery	1235	123	74·9	19
Abdominal aorta	1174	110	90·5	4
Femoral artery	1027	96	70·9	6
Popliteal artery	996	75	69·4	2
Perineurial arteriole	746	65	52·9	2
Femoral vein	218	29	43·7	6
Inferior vena cava	381	42	53·4	2

Mean arterial pressure and local flow rates and weights (F_1, F_2, W_1) were recorded using a microelectrode on the vessel wall at different sites in the vascular system. Each value represents the mean of several measurements.

DISCUSSION

H_2 saturated nerve may be cleared by a number of processes which together give rise to a biexponential clearance curve during the first 10–15 min of wash-out. If very fast and ultra-slow clearances are included in the analysis, typical clearance values (for the individual components) range from very slow (0–5), to slow (10–25), fast (70–100) and very fast (> 700 ml min^{-1} 100 g^{-1}). The clustering of rate constants around these values on a frequency distribution curve (Fig. 2) implies that certain physiological processes are responsible for all clearances in a certain range. Thus, it should be possible to assign some physiological significance to each clearance rate observed in a wash-out.

Nutritive blood flow (capillary flow) of around 15 ml min^{-1} 100 g^{-1} corresponds best to the slow component. This value is similar to values obtained from monoexponential clearance curves obtained from nerve (Low & Tuck, 1984), and correlates well with flows measured using iodoantipyrine (Myers, Mizisin, Powell & Lampert, 1982; Sladky, Greenberg & Brown, 1983) and microsphere methods (Tschetter, Klassen, Resch & Meyer, 1970).

Diffusion to air is a potential source of an artifact in our preparation, but the results of the diffusion experiment indicate that such a loss is very slow, equivalent to 2–3 ml min^{-1} 100 g^{-1} only. Such diffusion is unlikely to affect the assessment, therefore, unless NBF is very slow. However, theoretical considerations indicate that the rate of diffusion would be higher if the area of the oil bath were large or if it were very shallow. This latter circumstance can arise if the preparation is neglected and not kept covered with at least 3 mm of mineral oil.

Diffusion to areas of lower H_2 concentration is a potential source of error in tissues such as brain where there are two compartments with markedly different flow rates (Halsey, Capra & McFarland, 1977; Pearce & Adams, 1982; von Kummer, von Kries & Herold, 1986). There is no rapidly perfused tissue in the vicinity of nerves which

could set up concentration gradients as the wash-out proceeds and so distort the measurement of NBF. The surrounding tissues in our preparations include a small amount of loose connective tissue and skeletal muscle which have a resting flow rate of 6–11 ml min^{-1} 100 g^{-1} (Aukland *et al.* 1984; Sugimoto, Monafo & Eliasson, 1986). If it were to occur, intercompartmental diffusion would reduce the concentration gradient in nerve, giving rise to artificially low NBF values.

Spurious results would also be obtained if inadequate time were allowed for equilibration of H$_2$ concentrations prior to commencement of the wash-out phase. This is unlikely to be the case in our studies since loading is monitored as an increase in the polarographic current and the wash-out phase is not started until the nerve is fully saturated with H$_2$.

Clearance occurs through the walls of arterioles and venules as well as through capillaries (Stosseck, 1970), as evidenced by the fact that wash-out curves could be recorded from arterial and venous walls. A biexponential clearance is to be expected since an adventitial microelectrode measures the changes occurring in the arterial blood (which reflect the rapid pulmonary excretion of H$_2$) and also in the surrounding tissues, which behave as a single compartment perfused by that vessel. If, as we suggest, the NBF curves are influenced by arterial wall placement, then arterial adventitial recording is a good model for this situation. The low weight of the very fast component indicates that only a small portion of the sensing volume of the electrode is in contact with flowing blood, which indirectly indicates that the sensing volume is relatively small, probably having a radius comparable to the thickness of the arterial wall.

The very fast component remained rapid at all arterial sites down to the arteriolar level. This is consistent with its reflecting the 'arterial input function' or lung clearance of H$_2$ (Kety, 1951). It is not consistent with its representing local blood flow. The slow component was reduced as the calibre of the vessel decreased, approaching values comparable to the faster component of endoneurial H$_2$ clearance curves. A small volume surrounding an arteriole or other larger vessel will have a relatively high flow–volume ratio. As the microelectrode measures not absolute flow but the flow–volume ratio, it follows that a microelectrode recording close to a high flow vessel will yield a faster relative flow, i.e. larger k value or rate constant. This is supported by our observations (T. J. Day, unpublished observations) that biexponential curves (with a prominent fast component in the range of 70–120 ml min^{-1} 100 g^{-1}) may be produced by deliberately placing the electrode close to a visible epineurial vessel. In contrast, monoexponential curves or predominantly monoexponential clearances are most often associated with electrode placement in a portion of the nerve free of large visible vessels.

Its prompt clearance from arterial blood validates the assumption underlying the application of the Fick principle to H$_2$ clearance: that of negligible recirculation of H$_2$. When making measurements using nerve, and also in brain, it is common practice to exclude the first minute of the recording from the analysis in order to avoid any distortion of results due to recirculation of H$_2$ (Young, 1980; Pasztor *et al.* 1973). This data indicates that such treatment is more than adequate to ensure a zero arterial input function in the calculations.

Venous clearances may also be relatively fast, as shown in Table 3. These would

be expected to be multi-exponential as the venous blood reflects the flow in the tissues which it drains. If these tissues are heterogenous in their blood flow, this will be reflected in the number of exponents observed in the clearance curve. Recordings from epineurial veins yielded curves very similar to those recorded from an electrode deep in the endoneurium, again not unexpected as our calculations include the assumption that venous blood leaving the tissue has the same concentration of tracer as does the tissue. Accordingly, electrode placement close to a large vein would not be expected to show the same prominence of a fast component as a near-arterial location.

The nature of the vessels responsible for the fast component is clarified by our IVC occlusion and 5-HT infusion experiments. Acute elevation of venous pressure by ligation of veins or the Valsalva manoeuvre are established means of closing down arteriovenous fistulae (Sherman, 1963). A similar effect has been reported as a result of infusion of 5-HT (serotonin) (Saxena & Verdouw, 1982). The finding of a consistent decrease in the weight of the fast component (W_1) with IVC occlusion is strongly suggestive that much, although perhaps not all, of the faster clearance occurs through such shunt vessels. The finding that the rate of the faster clearance was not significantly slowed by IVC occlusion suggests that although AV shunts are likely to be present, they are not the sole contributors to this clearance rate. Although some shunts may remain patent and carry their original flow rate, their numbers are probably small.

In our experiments, 5-HT at lower concentrations was able to attenuate, and in some instances abolish, the fast component while there was a lesser effect on the slower wash-out rate. In group A, the reduction in the weight of the fast component (W_1) was similar to that seen in the IVC occlusion experiments ($47 \cdot 8$–$22 \cdot 6$ *vs.* $55 \cdot 1$–$15 \cdot 7$ %).

The marked reduction in nutritive flow in response to IVC occlusion and 5-HT infusion is of particular interest in view of recent morphological evidence which shows that rat neural capillaries are of a low-density large-diameter type (Bell & Weddell, 1984) and our findings that the microvasculature is physiologically a nutritive-capacitance system (Takeuchi & Low, 1987). Such a capacitance system would be unusually susceptible to IVC occlusion and, perhaps, 5-HT infusion.

Doses of 5-HT similar to those used in our experiments resulted in the closure of AV shunts in porcine skin with little discernible effect on nutritive flow in many tissues, although peripheral nerve was not examined (Saxena & Verdouw, 1985). The other cardiovascular effects of 5-HT in the rat were similar to those observed in pigs, rabbits and other species (Saxena, Forsyth, Johnston & De Werk, 1978; Takahashi, 1985). Thus, it is reasonable to assume that 5-HT closes AV shunts in the rat also. Such an effect would certainly explain the changes in the LDV signal used to monitor the effect of the infused 5-HT. If this is so, it provides further evidence for the fact that the fast component of the H_2 wash-out curve is strongly associated with AV shunt vessels, as suggested by the IVC occlusion experiments.

AV shunts have been demonstrated in the peri- and epineurium of peripheral nerve (Lundborg, 1975) and could be responsible for faster clearance by two mechanisms: firstly, the shunt vessel is a high flow vessel compared with a capillary and adjacent tissue would be washed out more quickly; secondly, shunting would result in dilution

of venous blood by that with a lower H_2 concentration. This would permit further H_2 extraction from the tissue by the venous blood as it coursed longitudinally through the nerve.

An alternative explanation, that of counter-current gas exchange between veins and arterioles has been proposed by Aukland and colleagues on the basis of recordings made in exercising muscle (Aukland *et al.* 1969). If that mechanism were to be operational in nerve, an increase in venous pressure would be expected to enhance the contribution of the shunt, not reduce it, as we found in our experiments.

The rate of the oxidation reaction at the electrode tip (which consumes H_2 and generates protons and electrons) can be estimated knowing that currents of 30 pA are commonly generated. We can calculate that 1.6×10^{-16} moles of H_2 are consumed per second while the tissue concentration of H_2 is approximately 8.4×10^{-8} mol cm^{-3}. As described in the Appendix, this results in a clearance óf approximately 0.2 ml min^{-1} 100 g^{-1}.

In conclusion, we consider the H_2 polarographic measurement to be a useful tool for the measurement of NBF provided that the physiological bases for the various components of the derived clearance curves are understood. We believe that the slower component, usually in the range of 5–25 ml min^{-1} 100 g^{-1}, represents nutritive blood flow, whereas the fast component (averaging 90–100 ml min^{-1} 100 g^{-1}) represents flow in subcompartments adjacent to AV shunts and possibly also in arterioles. Diffusion to air is a slow phenomenon (2–3 ml min^{-1} 100 g^{-1}) and is unlikely to contribute significantly to an estimation of NBF. Oxidation of H_2 at the electrode tip is even slower. Therefore, it is possible to separately examine nutritive and non-nutritive blood flow in nerve by application of multi-compartmental analysis. It is possible to ignore the fast component and concentrate on the slow phase when using H_2 wash-out techniques to assess nutritive nerve blood flow. Conversely, if AV shunts are being studied, then only the fast component need be considered.

<div align="center">APPENDIX</div>

<div align="center">*Mathematical modelling*</div>

Contribution of H_2 diffusion to wash-out curve

Hydrogen clearance from nerve by diffusion to air through oil is expected to follow an exponential course, according to Fick's First Law of Diffusion. Consider radial diffusion from a cylindrical nerve segment (radius a, length l) through a medium with diffusivity D, over a distance R. However, diffusion occurs only in a segment of angle θ bounded by the edges of the well (Fig. 7). The concentration at the surface of the nerve (i.e. $r = a$) is the concentration in nerve (C_n) multiplied by the oil–tissue partition coefficient (λ). The concentration at the surface of the oil (C_R) is taken to be zero, since the oil–air partition coefficient is so low ($\lambda = 0.018$) (CRC Handbook of Chemistry and Physics, 1986).

From Fick's Law of Diffusion, where Q is the amount of hydrogen in the nerve,

$$\frac{dQ}{dt} = DA\frac{dC}{dr}$$
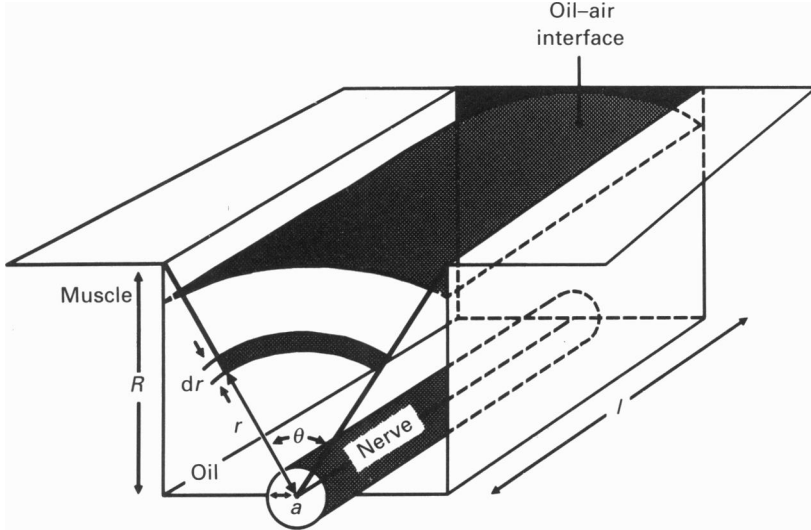
$$= D(\theta rl)\frac{dC}{dr};$$

Fig. 7. Model for calculation of diffusion-related clearance. Schematic diagram of the model used to calculate the H$_2$ clearance attributable to diffusion to air. A nerve of radius a and length l sits at the base of a well of depth R, whose walls subtend an angle θ at the nerve. The well is filled with oil and its walls are considered to be impermeable.

upon rearrangement,

$$\frac{dQ}{dt}\frac{1}{r} = (D\theta l)\frac{dC}{dr}.$$

Integrating with respect to the radial coordinate r gives

$$\int_a^R \frac{dQ}{dt}\frac{1}{r}dr = D\theta l \int_a^R \frac{dC}{dr}dr,$$

which becomes
$$\frac{dQ}{dt}\ln\frac{R}{a} = D\theta l(C_R - C_a).$$

Since $C_R = 0$,
$$\frac{dQ}{dt} = -D\frac{\theta l}{\ln(R/a)}C_a$$

which is equal to
$$-\frac{D\theta l\lambda}{\ln (R/a)}C_n.$$

Since the volume of nerve is known ($\pi a^2 l$),

$$\frac{dC_n}{dt} = -\frac{D\theta\lambda}{\pi a^2 \ln (R/a)}C_n.$$

The solution of this equation is

$$C_n = C_n(0)\exp^{-kt}, \text{ where } k = \frac{D\theta\lambda}{a^2 \pi \ln (R/a)}.$$

Measurements of nerve radius, oil bath depth and well dimensions were obtained directly from the experimental preparation. The oil–water partition coefficient was acquired from tabulated values (Lawrence, Loomis, Tobias & Turpin, 1946). The diffusion coefficient for H_2 in mineral oil has not been measured, to our knowledge. Since the diffusivity of H_2 in oil is related to that in water by the ratio of the viscosity of oil to the viscosity of water (Stokes–Einstein relationship, Atkins, 1978), it was possible to calculate the diffusivity of H_2 in oil using tabulated values for the diffusivity of H_2 in water (CRC Handbook of Chemistry and Physics, 1986) and the viscosity of mineral oil (US Pharmacopoeia, 1985). The following values were obtained: $D = 1.7 \times 10^{-6}$ cm^2 s^{-1} = 1.04×10^{-4} cm^2 min^{-1}; $\lambda = 3.1$; $R = 0.5$ cm; $a = 0.05$ cm; $\theta = 2 \tan^{-1}(0.3/0.5) = 1.08$ rad.

The rate constant can be calculated to be 0.019 min^{-1}, which corresponds to a flow of 1.9 ml min^{-1} 100 g^{-1}, which compares very well with the empirical value.

Rate of H_2 consumption by electrode

The polarographic reaction at the tip of the microelectrode may be represented as $H_2 \longrightarrow 2\,H^+ + 2\,e^-$, which commonly yields a current of 30 pA when 10 % H_2 is inspired by the rat. The rate of H_2 consumption can be estimated using the following equation:

$$\frac{dQ_n}{dt} = \frac{I}{2F},$$

where I is the current generated and F is the Faraday constant ($F = 9.6 \times 10^4$ C mol^{-1}). Thus $dQ_n/dt = 1.6 \times 10^{-16}$ mol H_2 s^{-1}. The tissue concentration of H_2 at full saturation is related to the P_{H_2} by the Bunsen solubility coefficient (α) and the nerve–water partition coefficient (λ). The latter could not be found in standard texts or references but was determined using our experimental preparation and measuring the H_2 concentrations with a H_2-sensitive microelectrode. The partition coefficient was found to be 1.17 ± 0.07 ($n = 13$) (T. J. Day, unpublished data). Thus for $P_{H_2} = 0.1$ atm, $\alpha = 7.14 \times 10^{-4}$ mol l^{-1} atm^{-1} (Biological Handbooks, 1970) and $\lambda = 1.17$, the tissue concentration of H_2 will be 8.4×10^{-5} mol l^{-1} = 8.4×10^{-8} mol cm^{-3}.

If it is assumed that the polarographic clearance of H_2 follows an exponential function, then $dQ/dt = -c[H_2]$, where the constant $c = (dQ/dt)/[H_2] = 1830\ \mu$m^3 s^{-1}. The above equation can be rewritten as $d[H_2]/dt = -(c/V)\,[H_2]$ where V is the volume 'sensed' by the electrode. The rate constant of H_2 clearance $k = c/V = 1830/V$. This sensing volume has been estimated by different investigators to have a radius of from 100 μm to 2 mm (von Kummer & Herold, 1986; Meyer, Fukuuchi, Kanda, Shinazu & Hashi, 1972; Halsey *et al.* 1977), with a single report of a 5 μm dimension (Betz, 1969). If we take the sensitive volume to have a radius of 250 μm (as an average figure), then the sensing volume $V = 6.5 \times 10^7\ \mu$m^3. Thus, the degree of H_2 clearance attributable to polarographic oxidation is approximately 0.17 ml min^{-1} 100 g^{-1} which is negligible when compared with physiological rates.

Multi-compartmental approach to H_2 clearance

If j parallel compartments are considered, each with concentration C_j, volume V_j and perfused by one vessel at flow rate F_j, then for each compartment the flux is:

$$\begin{aligned} dQ_j/dt &= -F_j\,C_j, \\ dC_j/dt &= -(F_j/V_j)\,C_j \end{aligned} \tag{1}$$

for which the solution is:

$$C_j = C_0 \exp\left(-F_j/V_j\right)t. \tag{2}$$

If total flux is considered and the principle of conservation of mass is applied, then:

$$dQ/dt = dQ_1/dt + dQ_2/dt + dQ_j/dt. \tag{3}$$

From eqn (1) it follows that:

$$dQ/dt = -F_1 C_1 - F_2 C_2 - F_j C_j. \tag{4}$$

From eqn (2) we have:

$$dC/dt = -(F_1/V_T) C_0 \exp\left(-F_1/V_1\right)t \\ -(F_2/V_T) C_0 \exp\left(-F_2/V_2\right)t - (F_j/V_T) C_0 \exp\left(-F_j/V_j\right)t, \tag{5}$$

where V_T = total volume of compartments. Solving this equation for C (the overall concentration) yields:

$$C = C_0[(V_1/V_T) \exp\left(-F_1/V_1\right)t + (V_2/V_T) \exp\left(-F_2/V_2\right)t \\ + (V_j/V_T) \exp\left(-F_j/V_j\right)t + 1 - (V_1 + V_2 + V_j)/V_T]. \tag{6}$$

Since V_T is the sum of the volumes of each compartment, then the last expression becomes 0, and:

$$C = C_0[(V_1/V_T) \exp\left(-F_1/V_1\right)t \\ + (V_2/V_T) \exp\left(-F_2/V_2\right)t + (V_j/V_T) \exp\left(-F_j/V_j\right)t], \tag{7}$$

i.e. a multi-exponential function where the exponents of the various components represent the relative flows (flow per unit volume) in the individual compartments. Thus, if a system comprises a rapidly cleared compartment ($F/V = 1\cdot2$) and a more slowly cleared compartment ($F/V = 0\cdot2$), and the electrode is placed such that 70% of its sensing volume is occupied by the faster cleared compartment, then the clearance equation will be:

$$C = C_0[0\cdot7 \exp\left(-1\cdot2t\right) + 0\cdot3 \exp\left(-0\cdot2t\right)].$$

Similarly, if the slow compartment occupies most (e.g. 95%) of the volume, implying that the faster compartment is more distant, then the equation will read:

$$C = C_0[0\cdot05 \exp\left(-1\cdot2t\right) + 0\cdot95 \exp\left(-0\cdot2t\right)].$$

The exponents are the same but the weights are quite different.

Any number of components can be combined in different proportions depending on how close the electrode is to the various compartments. These components need not represent anatomically discrete compartments since different physical processes within the same space may mimic an apparent additional compartment. The effect of three such combinations is shown in Fig. 8. The curve is multi-exponential and similar in appearance to those recorded experimentally (see Figs 4 and 5).
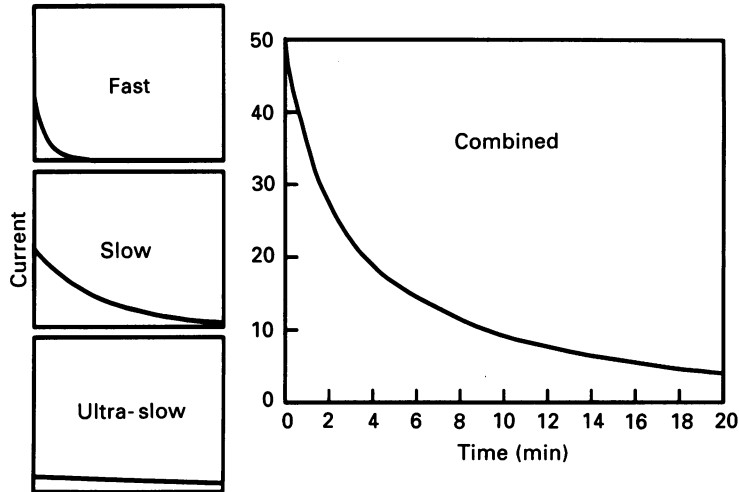
Fig 8. Model-generated triexponential H_2 clearance curve. The three components comprising the fast (AV shunt), the slow (nutritive) and ultra-slow (diffusion) are displayed on the left and the composite clearance curve on the right.

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