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Pressure injections of fluid in the nanoliter range via micropipettes

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The relationship between pressure, ejection duration and volume ejected was experimentally determined in vitro for micropipettes with different external tip diameters. The relationship between ejection duration and ejected volume is linear in the steady state (i.e. with ejection durations of 1 s or longer) and at sufficiently high pressures (above about 100 kPa) and for pipettes with a suffciently high hydrodynamic conductance (larger than 1 pl s^{-1} kPa^{-1} at 230 kPa). In this range, flows were found with low Reynolds numbers (smaller than 10), which is consistent with laminar flows. For all but the largest micropipettes, the relationship between pressure and ejected volume is alinear: the pipettes' apparent hydrodynamic conductances increase with increasing pressure. Micropipettes with apparent hydrodynamic conductances between 0.04 and 1400 pl s^{-1} kPa^{-1} (at 230 kPa) were tested. Duration–pressure combinations could be defined where the duration–volume relationship was either linear or monotonic. Such duration–pressure combinations were different for pipettes with different apparent hydrodynamic conductances. A quick method is described to measure the pipette's apparent hydrodynamic conductance at the pressure used, corrected for the fluid's viscosity. Measurement of this conductance permits predictable injections of known volumes of fluid in the range of 100 pl to 1 μl with a precision of 10–20%.

Introduction

The controlled, reproducible administration of known amounts of compounds in the picomole/nanoliter range is a requirement for several types of experiments, including intracellular staining and extracellular administration of biologically active compounds. In most studies, the compounds are administered by ionophoresis via micropipettes, but administration by pressure injection is the obvious alternative for compounds that are poorly soluble in water or not electrically charged (McCaman et al., 1977; Källström and Lindström, 1978; Sakai et al., 1979; Palmer et al., 1980;

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Dray et al., 1983). The number of investigators using pressure micro-injections is increasing (Walker et al., 1982; Spuhler et al., 1982; Palmer et al., 1982a, b; Nowak and MacDonald, 1982, 1983; Gold and Martin, 1983). The major drawback, however, of the pressure micro-injection technique was that the volume of the injected solution could not be controlled reliably (Krnjević et al., 1963; Obata et al., 1970; Kelly, 1975). At best, a poor correlation was found between the pipettes' external tip diameters or electrical resistances on the one hand, and the amounts released from these pipettes on the other. By definition, however, the amount ejected from a micropipette by pressure is determined by the pipette's hydrodynamic properties (e.g. Tritton, 1977) rather than its electrical or geometrical properties. The relationships between pressure, ejection duration, ejected volume and external tip diameter of micropipettes are reported in the present study. It is demonstrated that measurement of the pipette's apparent hydrodynamic conductance permits the administration of known volumes of fluid in the range of 100 pl to 1 μl with a precision of 10–20%.

Methods

Pressure injection apparatus

The pressure was applied to the micropipette according to the procedure of Palmer et al. (1980) and of Dray et al. (1983). In short, compressed nitrogen gas (300 kPa) was led into an apparatus containing a pressure-reducing valve and a timing valve (Medical Systems Corp., PPM-2); it was then applied to the micropipettes via high pressure tubing (length 300 cm, i.d. 3.0 mm) and a final soft tubing (length 15 cm, o.d. 2.5 mm, i.d. 1.0 mm).

Pipettes

The pipettes were pulled from glass tubes without filaments (Clark GC120, 1.2 mm o.d., 0.69 mm i.d.). Nitrogen gas (230 kPa) was applied to the unfilled pipette whose tip was immersed into water; when no air bubbles were formed, the pipette was called ‘clogged’. Clogged pipettes were broken under microscopic control, until they were unclogged. By this procedure, unclogged pipettes were obtained that had external tip diameters between 1.1 and 23 μm. Thereafter they were filled with distilled water and tested. All micropipettes remained intact at the highest pressures used (230 kPa, this study, or even 4 MPa, Källström and Lindström, 1978).

Measurements

The volumes ejected were measured with the microdrop technique (McCaman et al., 1977, Sakai et al., 1979). In short, the pipette was held under an angle of 45° with the horizontal plane, and its tip was immersed into mineral oil. The diameters of the ejected water droplets were measured with a microscope (magnification 40 ×) and a calibrated ocular micrometer. After each ejection, the diameter of the droplet was determined, the droplet was released, and a following droplet was ejected and measured. Linearity between parameters was checked in linear graphs and by linear regressions, and in double-log graphs and by double-log regressions.

Apparent hydrodynamic conductance

The apparent hydrodynamic conductance (G_h, in pl s^{-1} kPa^{-1}) was calculated. G_h appeared to be a function of the pressure, being most stable at high pressures (see below). To characterize pipettes, the value of $G_h(p)$ at a pressure of 230 kPa was used; this was the highest pressure that could be applied with the apparatus used. This value was called G_h^*. The smallest amount of fluid measurable with the equipment used was 10 pl. From 'clogged' pipettes no measurable amount of water (< 10 pl) was ejected in 20 s at a pressure of 230 kPa. Consequently, 'clogged' pipettes have G_h^* values smaller than 0.002 pl s^{-1} kPa^{-1}.

Results

Reliability

The reproducibility of the ejections was tested at a pressure of 230 kPa. Five series of five ejection durations each were applied with 30-min intervals between the series. The mean standard deviations in the ejected volumes were 8% (range 6–10%). This was in agreement with an estimated error of 9% in the measurement of volumes

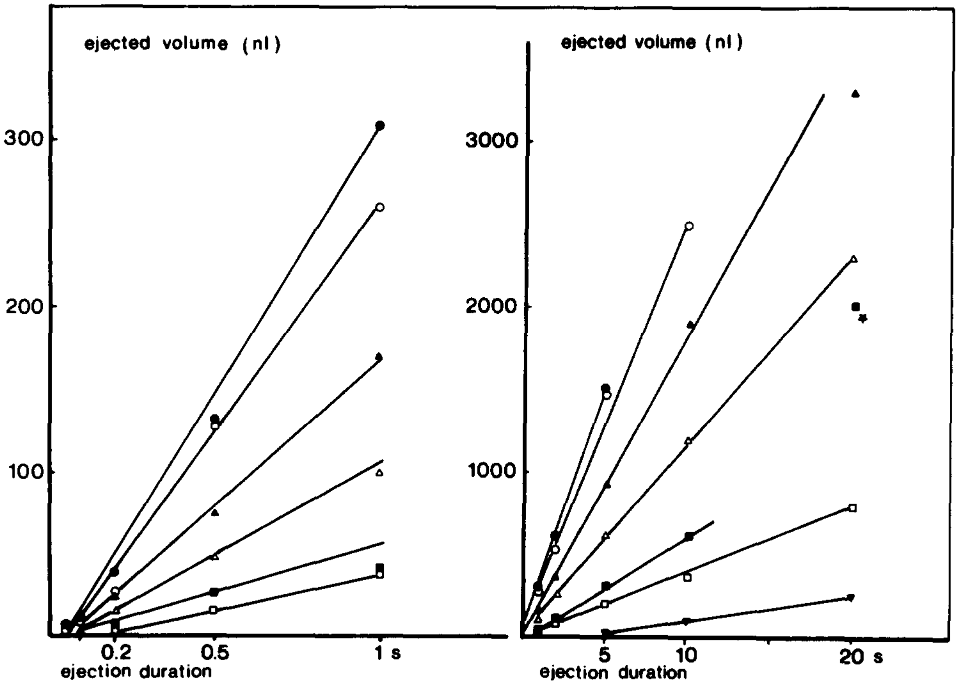

Fig. 1. Relationship between ejection duration and ejected volume for various pressures (data from a pipette with $G_h^* = 1400$ pl s^{-1} kPa^{-1}, linear graphs). Left: durations shorter than 1 s. Right: durations longer than 1 s. Pressures used: ▼ 18 kPa; □ 34 kPa; ■ 55 kPa; △ 95 kPa; ▲ 141 kPa; ○ 200 kPa; ● 223 kPa.

larger than 100 pl, but for volumes smaller than 100 pl, the error in the volume increased to 30%. An error of 10% applied for pipettes with G_h^* larger than 1 pl s^{-1} kPa^{-1}. Pipettes with a lower G_h^* value did not perform reliably with pressures lower than 230 kPa, and therefore their G_h value could not be determined reliably. In measurements of the ejected volumes at series of increasing and decreasing pressures no hysteresis was found, but only small variations within the error range. Moreover, the value of G_h at various pressures from several experiments with the same pipettes were the same, except sometimes for the lowest pressures.

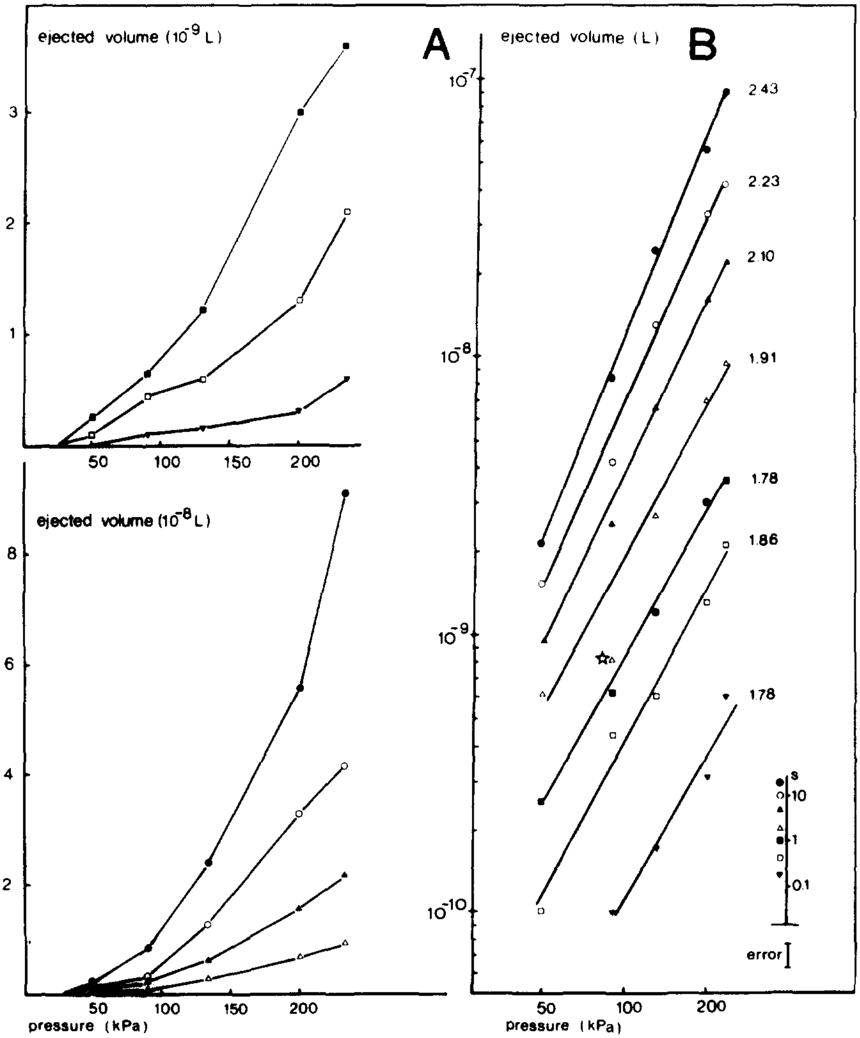

Fig. 2. Relationship between pressure and ejected volume at various ejection durations ranging between 0.2 and 20 s (data from a pipette with $G_h^* = 19$ pl s^{-1} kPa^{-1}). A: linear graphs. Above: durations shorter than 1 s. Below: durations longer than 1 s. B: double-log graphs of the same data; the numbers to the right are the gradients of the log–log regression lines.

TABLE I

MEAN GRADIENTS OF THE DOUBLE-LOG REGRESSION LINES BETWEEN PRESSURE AND EJECTED VOLUME FOR 4 PIPETTES WITH DIFFERENT VALUES OF G_h^*

Mean values for various ejection durations with the S.E.M. are given.

G_h^* (pl s^{-1} kPa^{-1})	Mean Gradients
0.3	2.49 ± 0.46
3.1	2.87 ± 0.20
19	2.01 ± 0.10
1400	1.16 ± 0.05

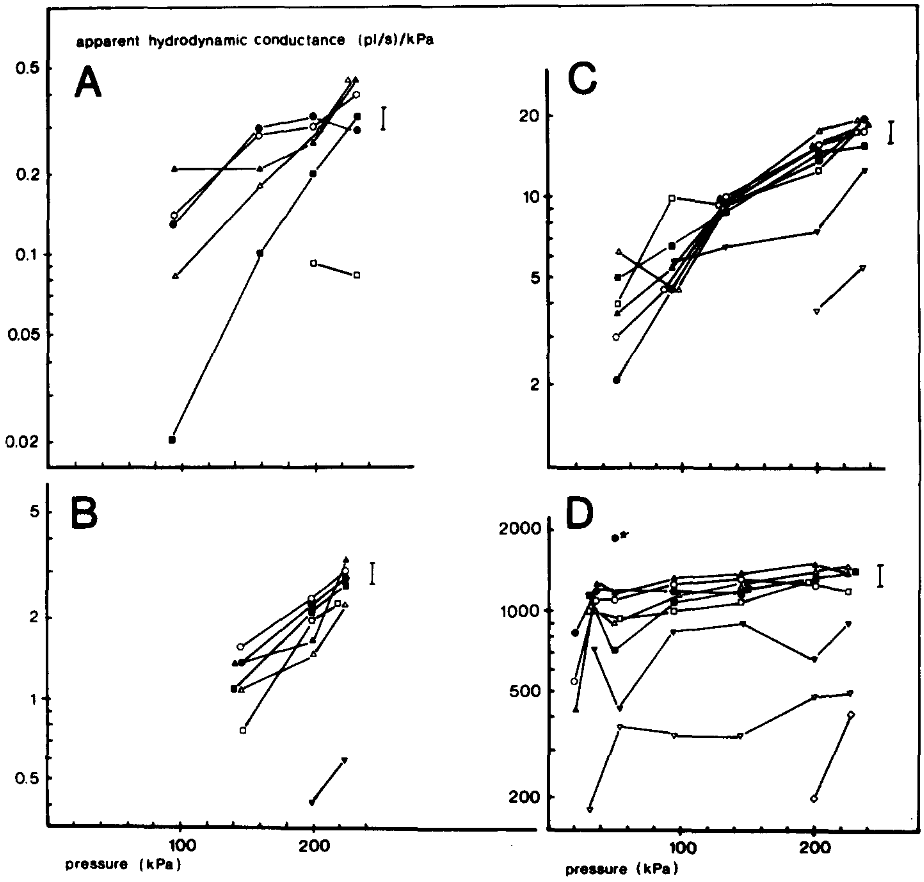

Fig. 3. Relationship between pressure and value of G_h at various ejection durations ranging from 0.05 to 20 s for 4 different pipettes with low (A) to high (D) values of G_h^* (C: pipette of Fig. 2; D: pipette of Fig. 1). Ejection durations: ◇ 0.05 s; ▽ 0.1 s; ▼ 0.2 s; □ 0.5 s; ■ 1 s; △ 2 s; ▲ 5 s; ○ 10 s; ● 20 s. The asterisk marks a measurement showing irregularity (see text).

Ejection duration and ejected volume

Graphs of the volumes ejected as a function of the ejection duration followed straight lines with an intercept on the horizontal axis (Fig. 1). With short ejection durations (depending on the pipette's G_h^* and on the pressure, see below), no droplet left the pipette.

Pressure and ejected volume

The relationship between pressure and ejected volume is shown in Fig. 2. At low pressures (depending on the pipette's G_h^*, and on the ejection duration, see below), no fluid was ejected. For higher pressures, the ejected volume was monotonicly rising with increasing pressures, but the relationship was alinear. Only for pipettes with the highest G_h^* values, the relationship between pressure and ejected volume was linear for pressures between 50 and 230 kPa, and ejection durations longer than 1 s. This is also expressed in the gradients of the double-log regression lines (cf. Fig. 2B and Table I).

Ejection duration, pressure and apparent hydrodynamic conductance

The relationship between ejection pressure and the pipette's G_h is shown in Fig. 3 for 4 pipettes with G_h^* values between 0.3 and 1400 pl s^{-1} kPa^{-1}. If the relationship between pressure and ejected volume were linear, the pipette's G_h must be indepen-

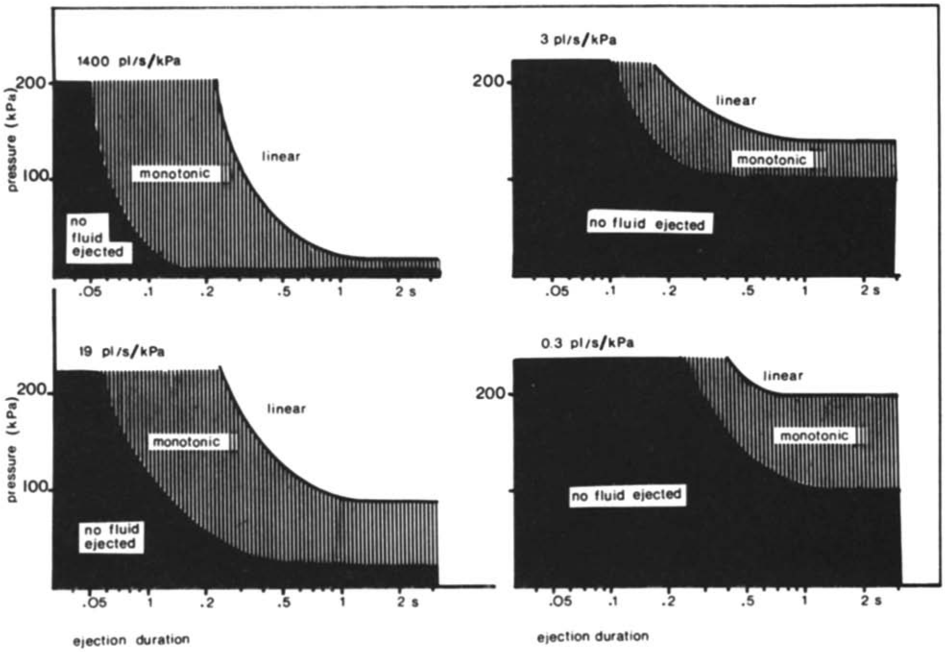

Fig. 4. Duration–pressure combinations where the duration–volume relationship is either linear or monotonic, or where no fluid is ejected, for pipettes with values of G_h^* ranging from 0.3 to 1400 pl s^{-1} kPa^{-1}.

dent from the pressure. The value of G_h, however, generally increased with increasing pressures; this increase was reproducible for the various pipettes and the various ejection durations. An increase of the pressure with a factor 2 often increased the pipette's G_h with a factor 2 to 3. Only for pipettes with the highest G_h^* value, the value of G_h was constant within the error range for pressures between 50 and 230 kPa and ejection durations of 1 s or longer (Fig. 3D).

Applicable ejection duration and pressure ranges

Fig. 4 gives a survey of the pressure–duration combinations for pipettes with various G_h^* values, where the duration–volume relationship was either linear or monotonic, or where no fluid was ejected. The relationship was called 'linear' as long as the points followed the double-log regression lines under an angle of 45°.

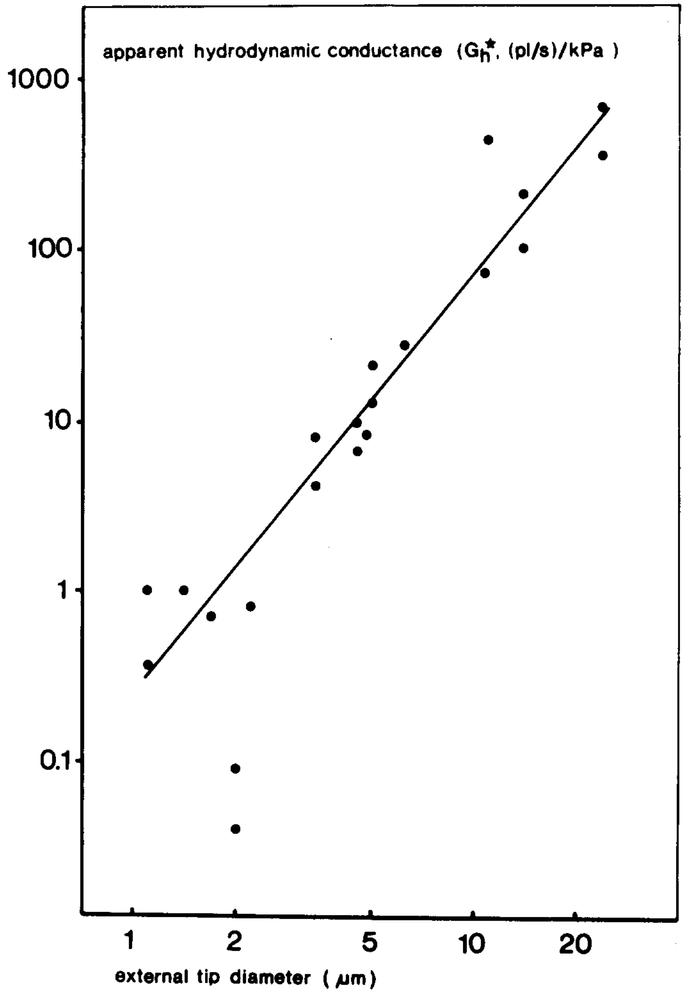

Fig. 5. Relationship between external tip diameter and the value of G_h^* for 21 pipettes. Indicated is also the log–log regression line, calculated for all pipettes.

External tip diameter and hydrodynamic conductance

The relationship between the external tip diameter of 21 pipettes and their G_h^* values is shown in Fig. 5 (log-log plot) with the double-log regression line ($\rho = 0.899$). Given an external tip diameter, the G_h^* can be estimated only with a very large error (double-log $\sigma_y = 1.2$, so the 'standard deviation' in G_h^* is a factor 16). The largest fluctuations are due to pipettes with G_h^* smaller than 0.1 pl s^{-1} kPa^{-1}, which have external tip diameters smaller than 2.5 μm. For pipettes with external tip diameters larger than 2.5 μm, the values of G_h^* can be estimated from the value of the external tip diameter with a reduced but still large error (double-log $\sigma_y = 0.71$, so the spread in G_h^* is a factor 5.1).

Irregularities

Some irregular measure points are indicated in the graphs by asterisks. Irregularities were encountered especially with pipettes with the lowest G_h^* value. Such pipettes often became clogged, or their G_h value showed large fluctuations. As a rule, however, the pressure micro-injections in the nanoliter range were reproducible.

Discussion

The pipette's apparent hydrodynamic conductance

The pipette's hydrodynamic properties are the relevant parameters for quantitative pressure micro-injections. The value of G_h at various pressures appeared to be reproducible, and G_h^* was strongly correlated with the external tip diameter. The value of G_h is corrected for the fluid's viscosity and for the pressures used, when the pipette's G_h is measured with the pressures and fluid that actually will be used (therefore it is called *apparent* hydrodynamic conductance). Such measurement can be done quickly by the droplet method.

Linear duration–volume relationships

The linearity of the duration–volume relationship as described in earlier papers is presently confirmed (McCaman et al., 1977; Sakai et al., 1979; Palmer et al., 1980; Dray et al., 1983). The intercept in the horizontal axis of the duration–volume curves appeared to be dependent of the pipette's G_h^* (cf. Fig. 4). Doubling the duration doubles the ejected volume at ejection durations longer than 1 s, because the influence of the above-mentioned intercept is negligible for such durations. This is in agreement with the data of Sakai et al. (1979) and Palmer et al. (1980), who only used ejection durations longer than 1 s.

Reynolds numbers

In hydrodynamics, a useful parameter is the 'Reynolds number' (R_e, cf. Tritton, 1977): it is the ratio between inertia and viscous forces for a given fluid flow:

$$R_e = \frac{\text{inertia forces}}{\text{viscous forces}}$$

For flows through pipes, the value of R_e is determined by:

$$R_e = \frac{u_{av} d_i}{\nu} = \frac{4V_e}{\pi \nu t_e d_i} = \frac{4G_h p}{\pi \nu d_i}$$

(in which u_{av} is the average flow speed in m/s, d_i is the internal pipe diameter in m, ν is the kinematic viscosity, V_e is the ejected volume in m^3, p is the ejection pressure in Pa, t_e is the ejection duration in s, and G_h is the apparent hydrodynamic conductance in $m^3\ s^{-1}\ Pa^{-1}$).

When the value of R_e is low (< about 2000), the flow is laminar, while turbulent flows are found at high values of R_e (> about 4000). For laminar flows, the mass passing per unit time through the pipe is proportional with the pressure (Tritton, 1977, p. 10). For turbulent flows, the mass flux is proportional with the square root of the pressure.

Laminar flows

The values of R_e were calculated, but only for those ejection durations where the pressure–G_h curves overlapped (cf. Fig. 3). The internal tip diameter was assumed to be half of the external tip diameter, which was the ratio before pulling. The estimated R_e values are plotted as a function of the pressure for 4 different pipettes

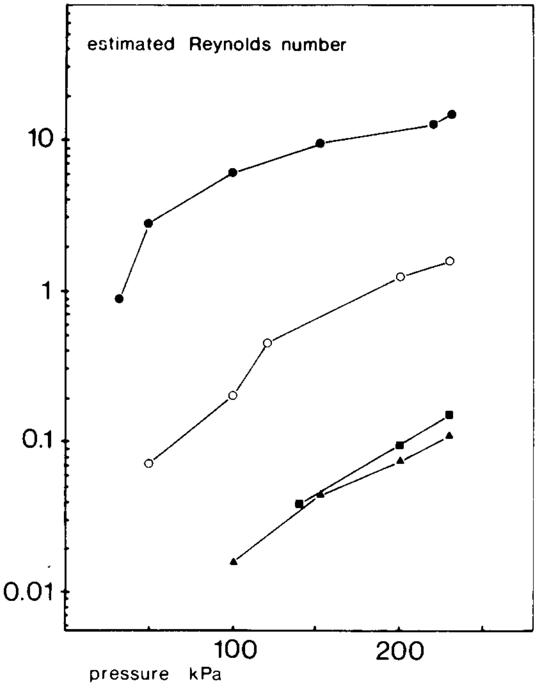

Fig. 6. Relationship between the pressure and the estimated Reynolds number for the 4 pipettes of Fig. 3. The internal tip diameter is assumed to be half of the external tip diameter. ▲: 0.3; ■: 3.1; ○: 19 and ●: 1400 pl s^{-1} kPa^{-1}

(Fig. 6). The Reynolds numbers of the flows used are smaller than 20, which is consistent with laminar flows through the pipettes. One pipette (with $G_h^* = 12$ (pl s^{-1} kPa^{-1}) was filled with ink, and about 1 nl was ejected in water; the ejected ink immediately dispersed like a cloud, and no jet flow was present, which is again in favor of low R_e flows and against turbulent flows (Tritton, 1977, p. 229).

Pressure–volume relationships

The findings above indicate that the flow through the pipettes is laminar, and therefore the relationship between pressure and volume ejected was expected to be linear (cf. Tritton, 1977). The relationship between pressure and ejected volume found in this study, however, is alinear except for the largest pipettes. We can only offer speculative explanations for such alinear pressure–volume relationships. The force due to the pressure on the water column in the pipette's tip is small for the internal tip diameters and the flows used in this study, so other forces like the adhesion between water and the pipette's glass wall might be relevant, and these might cause deviations from linearity in the pressure–volume relationship. The alinearity in the pressure–volume relationship is not due to surface tension in the ejected water droplets; if surface tension has a measurable influence, not only the pressure–volume relationships, but also the duration–volume relationships must be alinear, which is not the case. Other authors have however reported a linear relationship between pressure and volume ejected (McCaman et al., 1977; Palmer et al., 1980; Dray et al., 1983), but stricter controls for linearity have been used in the present study. Given the presently found alinearity, the value of G_h must be determined for the various pressures used, if one wants to change the dose by changing the pressure. In pressure ejection studies, it has become a bad habit to express the dose in units of pressure (Palmer et al., 1982a, b; Spuhler et al., 1982; Walker et al., 1982; Wuerthele et al., 1982; Gold and Martin, 1983), which is comparable to expressing ionophoretically injected amounts in voltages. The dose in moles is the relevant parameter; the concentration in the pipette, the injection pressure and the pipette's G_h at that pressure must be known to determine the dose.

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References

Dray, A., Hanley, M.R., Pinnock, R.D. and Sandberg, B.E.B. (1983) A comparison of the release of substance P and some synthetic analogues from micropipettes by microiontophoresis or pressure, Neuropharmacology, 22: 859–863.

Gold, M.R. and Martin, A.R. (1983) Inhibitory conductance changes at synapses in the lamprey brainstem, Science, 221: 85–87.

Källström, Y. and Lindström, S. (1978) A simple device for pressure injections of horseradish peroxidase into small central neurones, Brain Res., 156: 102–105.

Kelly, J.S. (1975) Microiontophoretic application of drugs onto single neurons. In L. Iversen, S. Iversen and S.H. Snyder (Eds.) Handbook of Psychopharmacology, Vol. 2, Plenum Press, New York, pp. 29–67.

Krnjević, K., Mitchell, J.F. and Szerb, J.C. (1963) Determination of iontophoretic release of acetylcholine from micropipettes, J. Physiol. (Lond.), 165: 421–436.

McCaman, R.E., McKenna, D.G. and Ono, J.K. (1977) A pressure system for intracellular and extracellular ejections of picoliter volumes, Brain Res., 136: 141–147.

Nowak, L.M. and MacDonald, R.L. (1982) Substance P: ionic basis for depolarizing responses of mouse spinal cord neurons in cell culture, J. Neurosci., 2: 1119–1128.

Nowak, L.M. and MacDonald, R.L. (1983) Ionic mechanism of muscarinic cholinergic depolarization of mouse spinal cord neurons in cell culture, J. Neurophysiol., 49: 792–803.

Obata, K., Takeda, K. and Shinozaki, H. (1970) Electrophoretic release of γ-aminobutyric acid and glutamic acid from micropipettes, Int. J. Neuropharmacol., 9: 191–194.

Palmer, M.R., Wuerthele, S.M. and Hoffer, B.J. (1980) Physical and physiological characteristics of pressure microinjection of drugs from multibarreled pipettes, Neuropharmacology, 19: 931–938.

Palmer, M.R., Freedman, R. and Dunwiddie, T.V. (1982a) Interactions of a neuroleptic drug (fluphenazine) with catecholamines in the hippocampus, Psychopharmacology, 76: 122–129.

Palmer, M.R., Sorensen, M.R., Freedman, R., Olson, L., Hoffer, B.J. and Seiger, Å (1982b) Differential ethanol sensitivity of intraocular cerebellar grafts in long-sleep and short-sleep mice, J. Pharmacol. exp. Ther., 222, 480–487.

Sakai, M., Schwartz, B.E. and Woody, C.D. (1979) Controlled microrelease of pharmacological agents: measurements of volume ejected in vitro through fine tipped glass microelectrodes by pressure, Neuropharmacology, 18: 209–213.

Spuhler, K., Hoffer, B.J., Weiner, N. and Palmer, M. (1982) Evidence for genetic correlation of hypnotic effects and cerebellar Purkinje neuron depression in response to ethanol in mice, Pharmacol. Biochem. Behav., 17: 569–578.

Tritton, D.J. (1977) Physical Fluid Dynamics, Van Nostrand/Reinhold, New York.

Walker, J.M., Moises, H.C., Coy, D.H., Baldrighi, G. and Akil, H. (1982) Nonopiate effects of dynorphin and des-tyr-dynorphin, Science, 218: 1136–1138.

Wurthele, S.E., Yasuda, R.P., Freed, W.J. and Hoffer, B.J. (1982) The effect of local application of homocysteine on neuronal activity in the central nervous system of the rat, Life Sci., 31: 2683–2691.