Preparation of solutions for perfusion staining. 1. Staining solution. Dissolve 0.130 g of 2-carboxy-2′-hydroxy-5′-sulfoformazylbenzene (Zincon, cat No. 3750, Sigma Chemical Co., St. Louis, USA) in 2 ml 1 N NaOH and dilute to 100 ml with redistilled water. 2. Buffer. Dilute 213 ml 1 N NaOH to 600 ml with redistilled water. Dissolve 37.3 g KCl and 31.0 g H_3BO_3 in the solution and dilute to 1:1 with redistilled water. 3. Perfusion fluid. To 100 ml of solution 1 add 200 ml solution 2 (or enough to bring pH to 10.0).

Procedure for perfusion staining. Rats were deeply anesthetized with pentobarbital sodium and, after opening the thoracic cavity, a polyethelene cannula was inserted into the ascending aorta through the left ventricle. The rate of perfusion was adjusted to 8 ml/min. The right auricle was opened to allow a low-resistance return of the perfusion fluid from the head. The perfusion was discontinued after 10–15 min when oozing of the fluid was apparent from a cut made in the upper lip. The skull was opened, the brain removed carefully and immediately frozen on the freezing stage of a cold microtome (Kryotome Model 1310 K, Leitz, Fed. Rep. of Germany). Frozen sections 20 µm thick were obtained after proper trimming of the block. They were collected on glass slides and dried. Some of the frozen sections were further stained with the same buffered staining solution (pH 10) for 0.5 min, then rinsed with deionized water. Glycerol gelatin (Catalogue No. GG 1, Sigma Chemical Co., St. Louis, USA) was used as the mounting medium.

Microscopy and photomicrography. Sections were examined with an Orthoplan universal large field microscope (E. Leitz, Wetzlar, Fed. Rep. of Germany) and the photomicrographs of the appropriate regions (figure 2) were obtained using an Orthomat W automatic microscope camera (E. Leitz).

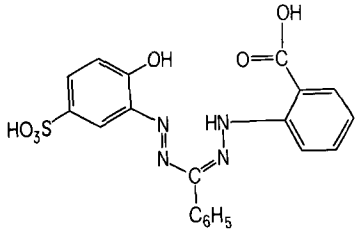

Fig. 1. The structural formula of 2-carboxy-2′-hydroxy-5′-sulfoformazylbenzene (Zincon).

Fig. 2. Photomicrograph of a frozen section of rat cerebrum showing zinc-Zincon reaction product in the form of dark blue granules and radiating fibres. Bar = 50 µm.

Results and discussion. The Zincon staining solution, which is deep red, forms a blue complex with zinc ions, especially in alkaline solutions (pH 9–10). Cold knife or cold microtome sections are undoubtedly best for the demonstration of natural tissue zinc. The zinc complex is stable over a pH range of 8.5–10, whereas the copper-Zincon complex is stable[5] in the pH range 5.0–9.5. This difference in effect of pH permits the ready detection of zinc at pH 9.5–pH 10. The dithizone method which is commonly used for histochemical demonstration of zinc is not so specific. The reddish dithizone staining solution forms a reddish-purple reaction complex with not only zinc but with a number of other heavy metals (Pb, Ag, Cu, Hg, Au, Cd) as well[3]. Atomic absorption spectrophotometric analysis of the brains obtained from the same group of rats has revealed that the Zincon method was highly sensitive to as low a concentration as 20 µg of zinc/g brain tissue. A unique characteristic of Zincon is its ability to diffuse uniformly to almost every part of the brain to react with zinc intravitally. The method described in this paper can be advantageously used to localize minute amounts of zinc rapidly and accurately.

6 M. Hasan, Ann. Ind. Acad. Med. Sci., *12*, 1 (1976).
7 R. M. Rush and J. H. Yoe, Analyt. Chem. *26*, 1345 (1954).

Intracellular pH of Limulus ventral photoreceptor measured with a double-barrelled pH microelectrode[1]

S. Levy and J. A. Coles[2]

Experimental Ophthalmology Laboratory, University of Geneva, 22, rue Alcide-Jentzer, CH-1211 Geneva 4 (Switzerland), and Department of Physiology, University of Geneva, Medical School, CH-Geneva (Switzerland), 23 September 1976

Summary. The intracellular pH of the Limulus ventral photoreceptor was measured with a double-barrelled pH microelectrode and found to be 7.01 ± 0.04 SE (n = 9).

In the course of studies on the light transduction mechanisms in Limulus ventral eye, experiments have been reported in which calcium buffers[3], pH buffers[3,4] and calcium sensitive dye[5] have been injected into the photoreceptor cells. Since the properties of these injected substances depend on pH, it is particularly interesting to have a value for the intracellular pH of these cells.

Materials and methods. The double-barrelled pH microelectrodes were made by a modification of the technique developed by Puccaco and Carter[6]. The principle consists of sealing a thin membrane of pH-sensitive glass over the tip of a micropipette made of a high electrical resistance, pH-insensitive glass. Although the technique worked well for single-barrelled pH microelectrodes, there was some

difficulty in obtaining double-barrelled ones. By using a different glass configuration and by improving 2 critical points: a) the sealing between the 2 barrels, b) the opening of the reference barrel, we successfully made double-barrelled pH microelectrodes.

Micropipettes were pulled from borosilicate theta capillaries (Hilgenberg-Glas, Glaswarenfabrik, D–3509 Malsfeld, Federal Republic of Germany) and ground on an air-driven plate of 0.3 μm rugosity (Stähli, CH–2542 Pieterlen, Switzerland) to have a bevel 4 μm wide (figure 1). A micropipette was then held vertically by a micromanipulator and a microscope focused on the tip. A pH membrane (about 0.08 μm thick) was held horizontally above the tip and heated by a small filament; simultaneously, the pipette was advanced upwards through the membrane so that a piece of membrane covered the final 100–200 μm of the pipette tip[6]. We found that the membrane sealed over the bevel better if the pressure inside the pipette was reduced by connecting it to a filter pump. Care was taken not to overheat the glass membrane. The pH glass used was an uranium-containing glass (SiO_2 61.5%, UO_2 2.1% MgO 8.1%, Na_2O 28.3%). Thanks to the theta configuration, the microelectrode could be filled directly with a hypodermic needle. The pH barrel was filled with magnesium acetate 1 M, and the reference side with KCl 2.5 M, KNO_3 0.5 M; an Ag-AgCl wire was placed in each barrel and sealed with dental wax.

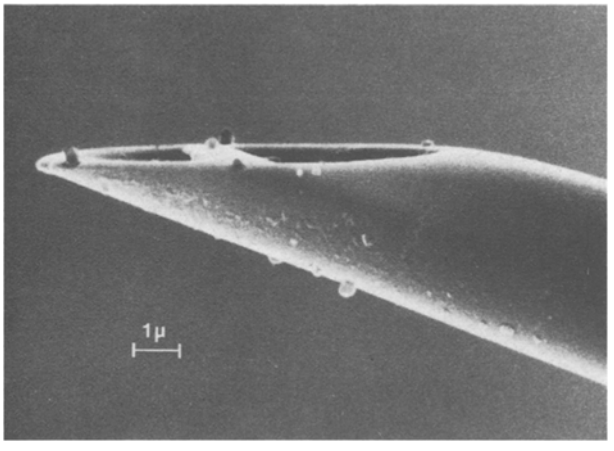

Fig. 1. Scanning micrograph of a theta glass micropipette after bevelling. The reference barrel is on the left; the pH barrel is on the right. The pH membrane is mounted afterwards. (The photograph was taken by Miss S. Voorhoeve using a technique developed by M. Baumann, Department of Histology, University of Geneva.)

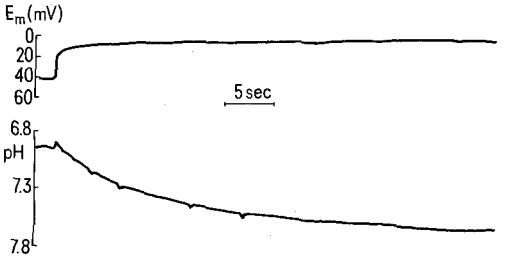

Fig. 2. Recording of the removal of the double-barrelled pH microelectrode from a Limulus ventral photoreceptor. The upper trace shows the membrane potential (Em) recorded by the reference barrel. The lower trace shows the potential difference between the 2 barrels and thus records pH. In this experiment, the artificial sea water had a pH of 7.64.

In order to have a working double-barrelled pH microelectrode, the reference side must be open; this was achieved by applying an AC voltage across the membrane on the reference barrel. The tip of the microelectrode was immersed in a satured NaCl solution and a variable 50 Hz voltage source (up to 200 V RMS) connected to the reference side through a 1 M Ω series resistor. The voltage was increased gradually until the current, monitored on an oscilloscope, suddenly increased. The pH barrel was kept connected to a high impedance amplifier and resistance measuring circuit to check that its membrane remained intact. The electrical resistance of the 2 barrels was then checked: about 90% of the electrodes had $5–30 \times 10^6$ Ω for the reference side and $1–5 \times 10^{11}$ Ω for the pH side. Electrodes with resistances in these ranges almost always responded to pH changes with 56–58 mV per pH unit. Each barrel was connected to an operational amplifier (Teledyne Philbrick 1029, 10^{13} Ω input impedance) with capacity neutralisation.

The ventral eye of Limulus was dissected out of the animal, treated with pronase, and mounted in a Sylgard 184 chamber[7]. The chamber was continuously superfused with artificial sea water (ASW) (NaCl 423 mM, KCl 10 mM, $MgCl_2$ 22 mM, MgSO4 26 mM, $CaCl_2$ 10 mM, Tris-Cl buffer 10 mM, pH 7.6–7.8). Light from a Xenon lamp was passed through an electromechanical shutter and was focused on the preparation. The measurements were performed in the dark; weak test flashes were presented to check that both barrels were recording the receptor potential (and hence were intracellular). The electrode was advanced towards the upper surface of the cell at about 45° with the sharp side of the bevel downwards.

Results and discussion. Penetration of the cell was achieved by momentarily overcompensating the input capacitance of the reference barrel[8]. The electrode was removed from the cell within 5 min and the pH was measured from pen recordings at entry and exit (figure 2). The mean value obtained for the intracellular pH was 7.01 ± 0.04 SE (n = 9). Microspectrophotometry of Limulus ventral photoreceptors injected with phenol red has been performed by J.E. Lisman and P.K. Brown and by J.E. Brown and P. K. Brown (personal communications) who obtained pH values of 6.8–6.9. Apart from small differences attributable to the high resistance of the pH barrel, the same intracellular receptor potential evoked by light was recorded from each barrel. This shows that the pH barrel entered the cell together with the reference barrel. We conclude that this double-barrelled electrode can be used for intracellular measurement of pH. The fact that the reference barrel can be electrically oscillated appears to be helpful for penetration of Limulus ventral photoreceptor cells and may perhaps be useful in other cases.

1 This work was supported by the Swiss National Science Foundation, grants Nos. 3.634.075 and 3.128.73.
2 Acknowledgments. We thank Drs L. R. Pucacco and N. W. Carter for technical advice and a gift of pH glass. We also thank Drs M. Tsacopoulos and F. Baumann for helpful discussion.
3 J. E. Lisman and J. E. Brown, J. gen. Physiol. *66*, 473 (1975).
4 J. A. Coles and J. E. Brown, Biochem. biophys. Acta *436*, 140 (1976).
5 L. H. Pinto, J. E. Brown and P. K. Brown, Biophys. J. *16*, 34a (1976).
6 L. R. Puccaco and N. W. Carter, Analyt. Biochem. *73*, 501 (1976).
7 J. E. Lisman and J. E. Brown, J. gen. Physiol. *59*, 701 (1972).
8 D. A. Baylor, M. G. F. Fuortes and P. M. O'Bryan, J. Physiol. *214*, 265 (1971).