observed for several minutes (Figure 1, B). In spite of the effect of erythro-L-BHGA, 150 pg L-glutamic acid applied in the same manner did not produce any effect on the PON. We could conclude by the microdrop application experiment that the inhibition produced by erythro-L-BHGA was due to the hyperpolarization of the PON membrane, and not due to synaptic noises.

The neuromembrane rectification was previously reported[9,10]. In the present study, we measured the I-V curve of the neuromembrane as an indicator of membrane resistance by applying a triangular current (hyperpolarizing, depolarizing and hyperpolarizing; almost 2 min a cycle). Figure 2 shows the change in the I-V curve of PON membrane caused by the bath application of erythro-L-BHGA. In this figure, the I-V curve obtained in the application of this substance at 3×10^{-5} g/ml (b) was superimposed on the I-V curve measured in the physiological state (a); in both of these I-V curves, the initial biopotential level (just before the transmembrane current application) was used as the common standard point. In the artificially (by the transmembrane current) hyperpolarized state of the neuromembrane, a clear decrease of membrane resistance was observed in the case of erythro-L-BHGA. In the artificially depolarized state, an elevation of firing level was observed under this substance.

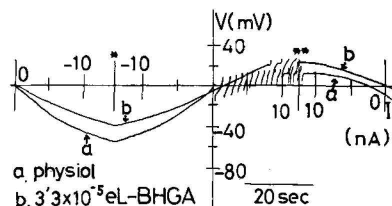

Fig. 2. Change of the current-voltage relationships (I-V curve) of the PON membrane caused by the application of erythro-β-hydroxy-L-glutamic acid (eL-BHGA). A triangular current (hyperpolarizing, depolarizing and hyperpolarizing) was applied to the soma to obtain the I-V curve. Ordinate: Biopotential shift produced by the transmembrane current (mV). Abscissa: Intensity of the transmembrane current (nA). From zero, the current was applied gradually until reaching 15 nA in the hyperpolarizing direction (asterisk). Then the current was adjusted to the depolarizing direction. After reaching about 12 nA in the depolarizing direction (2 asterisks), the current was adjusted again to the hyperpolarizing direction. a) I-V curve measured in the physiological state. b) I-V curve obtained 3 min after the bath application of eL-BHGA at 3×10^{-5} g/ml. The two I-V curves were superimposed, using the initial biopotential level (just before current application) as the common standard point. Note that the lower membrane resistance and the higher firing level were caused by the application of eL-BHGA in comparison with the physiological state.

SCHLEIFER et al.[11] reported the presence of threo-BHGA in the cell wall of a bacterium. OHARA et al.[12] also demonstrated the presence of threo-BHGA in the mammalian brain. We assume that erythro-L-BHGA is probably an inhibitory transmitter of the PON, or a partial agonist of an unknown transmitter which is not glutamic acid or aspartic acid. Previously we reported[4] the inhibitory effect of L-homocysteic acid and L-homocysteine sulfinic acid on the PON. If erythro-L-BHGA is a partial agonist of an inhibitory transmitter of the PON, there arises the question of whether erythro-L-BHGA and the two sulfur-containing amino acids are agonists of the same inhibitory transmitter.

Summary. We found a spontaneously firing neurone, inhibited by β-hydroxy(erythro)-L-glutamic acid, identified in the subesophageal ganglia of an African giant snail (*Achatina fulica* Férussac), although this neurone is not sensitive to L-glutamic acid. We suggest that β-hydroxy(erythro)-L-glutamic acid may be a putative inhibitory synaptic transmitter of the identified molluscan neurone.

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Use of a Tissue Culture Medium for in vitro Studies on the Ion Transport Capacity of Amphibian Epithelia

In most studies on the physiological role of the transport of ions across isolated epithelial membranes, simple electrolyte solutions have been used to incubate the preparations, to keep the concentration of the principal 'physiological' ions, such as Na^+, K^+, Ca^{++} and Cl^-, the total osmolality and pH within the range of values for the extracellular fluids of the species under study. In some cases, especially when mammalian tissues are used, a nutrient substance such as glucose is added to the basic electrolyte solution. These so-called Ringer solutions have proved satisfactory for most physiological experiments, provided that they are carried out within a short time of the isolation of the tissue. However, a decay of the transport capacities of isolated epithelial membranes is an almost constant finding, so that one can argue that such experiments are carried out in dying preparations. This argument is especially valid when applied to mammalian tissues, such as the small intestine, where the rate of decay may be very high from the mounting of the experimental set-up[1]. Although amphibian tissues are much less rapidly affected by the isolation procedures and keep their transport properties reasonably well, at least for a few hours, we decided to see whether the use of a more

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complex medium of the type used in cell and organ culture might improve the condition of 'physiological' preparations, at least to the extent of allowing their use for longer experimental periods.

The following experiments were designed to compare, by the classical *Ussing* technique[2], the basic transport properties of amphibian epithelial membranes incubated in tissue culture medium or in a Ringer solution. Suitable media for amphibian cell and organ culture are now well documented[3,4], and the medium used in this study, hereafter referred to as MEM, contained: 50% MEM (minimal essential medium, Eagle), 10% foetal bovine serum, 40% distilled water, 15 m*M* HEPES (zwitterionic buffer), 15 m*M* NaCl, 100 units/ml penicillin and 100 µg/ml streptomycin sulphate. The sodium chloride was added so that the total sodium concentration and the osmolality (220–225 mOsm) were the same as in the NaCl Ringer solution, which had the following composition: 114 m*M* NaCl, 2.5 m*M* KCl_2, 2.5 m*M* $CaCl_2$, 5 m*M* glucose and 1 m*M* *Tris*. The pH of both solutions was adjusted to 7.5 by adding a small amount of NaOH (to MEM) or HCl (to Ringer) . . CO_2-free air was bubbled through the chambers to stir and aerate the solutions. A drop of polyolic antifoam (Scientific Instruments, ref. 820) was added to the half-chambers containing MEM, to avoid the excessive formation of foam, and preliminary experiments showed that this had no effect on the parameters measured.

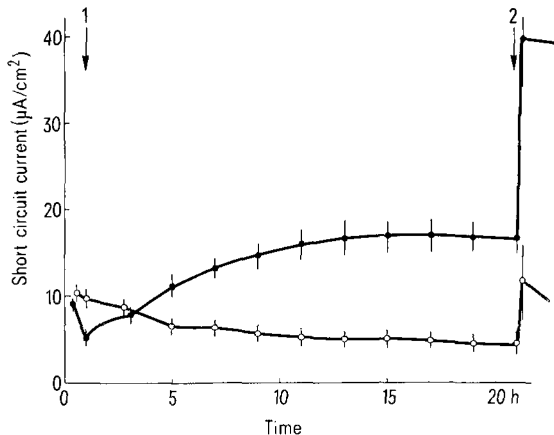

Fig. 1. Plot of mean values of SCC versus time in 2 groups of frog skin preparations. Open circles, skins bathed by NaCl Ringer; closed circles, skins where MEM was introduced in the half chamber bathing the corium (at arrow 1). Arrow 2, antidiuretic hormone in both groups. Means and SE. are represented. 11 experiments in each group.

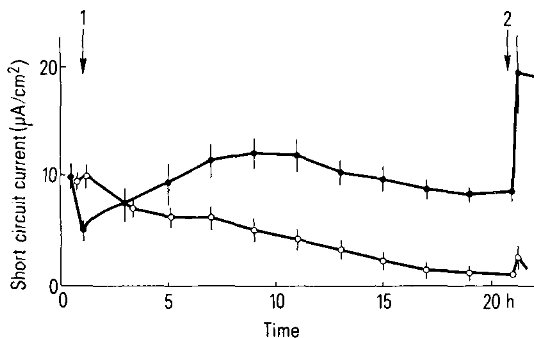

Fig. 2. The same as Figure 1, for groups of preparations of isolated frog skin epithelium. 12 experiments in each group.

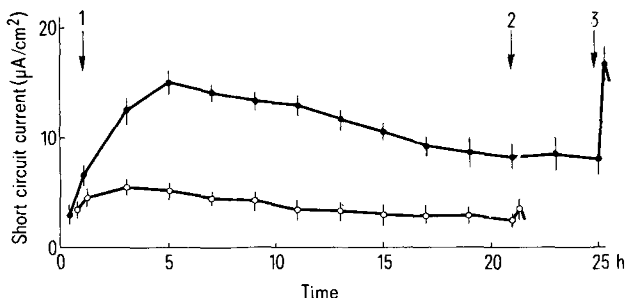

Fig. 3. The same as Figure 1, for groups of preparations of toad bladder. Arrow 2, antidiuretic hormone in the preparations incubated in Ringer. Arrow 3, antidiuretic hormone in the preparations incubated in MEM. 12 experiments in each group.

Three different preparations were used: whole frog skin and separated frog skin epithelium from *Rana ridibunda*, and toad bladder from *Bufo bufo*. After double pithing of the animals, pieces of frog abdominal skin or toad hemi-bladders were rapidly dissected and mounted as described below. The separated frog skin epithelium was obtained by a technique first described by Aceves and Erlij[5] and modified by Rajerison et al.[6], using the combined action of collagenase (40 U/ml) and hydrostatic pressure (4–6 cm H_2O). Once this stage was completed the same protocol was used in all the experiments. The preparations were mounted in Ussing-type chambers, with an exposed area of 3.1 cm² and volume of 4 ml on each side. Ringer was used for an equilibration period of 30–60 min. Fresh Ringer solution was then placed on both sides of the preparations randomly chosen as controls. For experimental preparations, MEM was introduced to bathe the corium face of skin and the serosal side of bladder and fresh Ringer was placed in the opposite half chamber. Short circuit-current (SCC), which is a function of the active sodium transport across these tissues, was automatically injected and recorded by means of a voltage clamp device and multichannel recorder. The open circuit voltage (OCV) was regularly checked, except during the night period. All the preparations were left overnight under short circuit conditions, and the next morning the incubating solutions were renewed. Antidiuretic hormone (Pitressin, Parke-Davis, 100 mU/ml) was added to the internal bathing medium 20–24 h after the preparations were set up.

Figures 1–3 show mean values of SCC taken at 2 h intervals from the continuous records of the control and experimental groups of preparations. Maximal values obtained after stimulation with antidiuretic hormone are also plotted. On the average, these graphs show that the mean values of SCC of the preparations incubated in MEM tended to increase in time, at least for a period of 5–8 h, while in all the control groups a deterioration of the SCC was noticeable either from the start of the experiments or after a 2–3 h initial period of slow increase. Looking into more detail at the evolution of the SCC and

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OCV of the preparations incubated in MEM, we could see that the introduction of this solution was followed by a gradual increase of the SCC and OCV values (preceeded, in frog skin and isolated epithelium, by a sharp, transient fall shown in Figures 1 and 2). In the experiments with whole frog skin, the upwards trend of the SCC continued for the next 10–12 h, after which a plateau was kept at a level of 16–17 $\mu A/cm^2$, which was 3–4 times higher than the control preparations at the same time, and twice as the initial values obtained with this structure. Open circuit voltages increased from 28 $\pm$ 2 mV at the outset of the experiments to 57 $\pm$ 3 mV at 20 h (in control preparations, OCV at 20 h was 13 $\pm$ 3 mV).

In the group of isolated epithelium of frog skin incubated in MEM, the SCC also increased steadily during the first 8 h, reaching a mean value of 12 $\mu A/cm^2$, which is more than twice the value obtained in the control group at the same time (Figure 2). After this period the SCC tended to decay, but the mean value at 20 h was still 8.6 $\mu A/cm^2$, in contrast with the almost complete deterioration of the control preparations.

In the case of the toad bladders, the very low initial values of the SCC increased very quickly after the introduction of MEM, the mean value reaching a peak of 15 $\mu A/cm^2$ at 4 h of incubation. From this time on, there was a downwards trend of the SCC, which tended slowly towards a plateau-like region at 8–9 $\mu A/cm^2$. The voltage values were initially very low (3 mV), rising to 10 mV at 2–4 h and keeping this value for the remaining period.

Stimulation by antidiuretic hormone. The difference between the control and experimental groups was also manifested in the intensity of the response to antidiuretic hormone. In fact, preparations incubated in MEM reacted to the addition of this hormone with an increase in the SCC which was of the order of 20 $\mu A/cm^2$ for whole frog skin, 10 $\mu A/cm^2$ for the isolated epithelium and 9 $\mu A/cm^2$ for toad bladder. In comparison the increase of the SCC in control groups was transitory and with a mean peak value of 7 $\mu A/cm^2$ for whole frog skin, and was negligible for frog skin epithelium and toad bladder. The degree of response to antidiuretic hormone in the experimental groups may also be compared with the results obtained in a previous work with preparations incubated in Ringer where the hormone was used at an early stage (4–6 h). The increase of the SCC was of the order of 15 $\mu A/cm^2$ for whole frog skin and 12 $\mu A/cm^2$ for the separated epithelium[7] and 9 $\mu A/cm^2$ for toad bladder (unpublished results).

In summary, considering that the SCC of frog skin and toad bladder is a function of the net sodium flux[2,8], these results show that there is a definite improvement in the sodium transport capacity of these structures when MEM is used. This improvement is patent either in the first phase of the experiments, when the increase in the SCC in the preparations incubated in MEM contrasts with the tendency to decay of the control ones, and in the long-term maintenance of the electrical parameters and the capacity to react to a physiological stimulus. Morphological studies should help to elucidate the effects of MEM in conserving the transport capacities of these isolated tissues.

Summary. The sodium transport capacity across frog skin and toad bladder in vitro is enhanced when a tissue culture medium is used to mount the preparations, instead of the classic Ringer solution. The response to antidiuretic hormone used 24 h after isolating the tissues is also higher in preparations incubated in the same culture medium.

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A Study of Micro-Circulation in Web of Frog (*Xenopus laevis* Daudin) by Using Laser Doppler Microscope

A method has been described to measure flow profiles in microscopic transparent tubes by the application of a laser technique (Laser Doppler microscope[1–4]). The method yields frequency distributions of flow velocities at various distances from the centre of the tube, An example of such a measurement on biological object is reported here. The velocity profile deviates slightly from a Newtonian parabolic profile.

A frog (*Xenopus leavis* Daudin) lay anesthetized in a shallow lucit tray containing a 0.1% urethane solution. A hind limb was hung up in the air and sustained with metal pins on a holder plate standing perpendicularly to the passage of the light beams. The holder plate had a small window through which the 2 laser beams were brought into the web. Both the tray and the holder were placed on a manipulator which was movable by the scale of 5 μm over a wide range (Figure 1). The greater part of the web and hind limb sustained, except the portion of observation (i.e. the passing area of the beams), was covered with a piece of wet gauge in order to prevent the frog from drying up. Although the breathing via the lung of the frog was not maintained artificially, it lasted throughout the experiments for 3 h under the above conditions.

Measured results of the blood flow velocity in a venule having a diameter of 70 μm are shown in Figure 2, where the ordinate and abscissa indicate the flow velocity of the blood and the appearing number of each velocity, respectively. The peak flow velocity decreased gradually

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