were then washed in 3 changes of Ringer's solution and the SCC in each half was measured. The SCC of half-hemibladders photolyzed under control conditions, i.e. without having been treated with an amiloride analog, was 137% of the SCC prior to photolysis (figure, A). The SCC of the other half-hemibladders, those portions not irradiated, was 106% of the previous value. This indicates that photolysis under these conditions does not inhibit the SCC, while the half-hemibladders protected from irradiation serve as acceptable controls for the irradiated halves. Since bromoamiloride was reported to inhibit irreversibly 30% of the SCC after UV-activation[4], we examined the effect of bromoamiloride on the SCC following photolysis as described above. The SCC of half-hemibladders exposed to bromoamiloride during photolysis was 60% of the SCC value preceding photolysis (figure, B). The unphotolyzed halves had an SCC of 112% of the previous value, indicating noncovalently bound bromoamiloride had been washed out of the preparations. Thus 40% of the SCC was irreversibly inhibited by this treatment, as reported by Benos and Mandel[4]. Although the concentration of bromoamiloride used to achieve this degree of irreversible inhibition was much higher than that used by Benos and Mandel, two aspects of our experimental design should make it superior to theirs. First, the radiation used in their study is of a wavelength generally destructive to proteins and second, the length of exposure to irradiation was shorter than in their study, with a resultant decrease in the amount of SCC remaining.

Using the same protocol, the effects of iodoamiloride were examined. The SCC of half-hemibladders photolyzed in the presence of iodoamiloride (100 µM and greater) was completely abolished in all trials (figure, C). At lower concentrations with equal lengths of photolysis, the inhibition was reduced. In the tissue-halves treated with iodoamiloride without photolysis, the SCC attained 114% of the pretreatment SCC following removal of the drug, indicating the reversible action of unphotolyzed iodoamiloride. Although the inhibitory potency of unphotolyzed iodoamiloride is less than that of bromoamiloride, the iodinated analog is more promising for photoaffinity labeling studies for at least two reasons: 1. Iodoamiloride appears to form a more highly reactive intermediate. This is important because relatively unreactive species are likely to attach covalently to numerous sites on the membrane. On the other hand, highly reactive species are likely to react with their specific binding sites before they dissociate and diffuse to other less specific sites. Furthermore, for derivatives which form highly reactive species, shorter times of photolysis will probably inactivate more sodium sites thus reducing the necessary amount of irradiation, which could cause considerable damage to proteins. 2. Monitoring the photoaffinity labeled molecule, which might be a relatively minor component of the membrane, requires a radioactive label of high sp. act. Iodoamiloride offers an excellent prospect, since it can be prepared with radioactive iodine of high (^{125}I, 2500 Ci/mmole) sp. act.

1 J.D. Bicking, J.W. Mason, O.W. Woltersdorf, Jr, J.H. Jones, S.F. Kwong, C.M. Robb, and E.J. Cragoe, Jr, J. med. Chem. *8*, 638 (1965).
2 P.J. Bentley, J. Physiol., *195*, 317 (1968).
3 A.W. Cuthbert, J. Physiol., Lond. *228*, 681 (1973).
4 D.J. Benos and L.J. Mandel, Science *199*, 1205 (1979).

The effects of sodium and amiloride on the motility of the caudal epididymal spermatozoa of the rat[1,2]

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Summary. The forward motility of the rat caudal epididymal spermatozoa has been studied in different Na^+ concentrations. When spermatozoa were suspended in a completely Na^+-free solution, the forward motility suffered a progressive fall and after 3 h was completely suppressed. This effect was fully reversible on resuspending the spermatozoa in a solution containing Na^+. Amiloride caused a fall in motility and the effect was similar to that of Na^+ removal. The inhibition by amiloride of the motility was concentration dependent and the dose response curve showed an IC_{50}-value of about 5×10^{-5} M. The role of Na^+ influx in the maintenance of sperm motility was discussed.

In the rat when the spermatozoa reach the cauda epididymidis, they are fully mature but are maintained in a quiescent state during storage. The caudal epididymal fluid contains a high concentration of potassium and a low concentration of sodium[3,4]. This results from the active absorption of sodium and secretion of potassium by the epididymal epithelium[5]. The high K^+/Na^+ ratio in the epididymal fluid may be responsible for maintaining the spermatozoa in an inactive state hence reserving energy for the vital processes of capacitation and fertilization. It has been reported that monovalent cations like sodium and potassium affect the motility of spermatozoa of many species[6,7]. However, the precise relationship between ion transport across the sperm membrane and sperm motility has not been explored. In this work, we have studied the effect of different sodium ion concentrations on the forward motility of the rat caudal epididymal sperm. Amiloride, a drug which is known to block Na^+ influx into cells[8] has also been used as a tool to investigate the role of sodium influx in sperm motility.

Methods. Spermatozoa were collected by flushing out the contents of the cauda epididymidis with sodium-free tris buffer after cannulation of the cauda epididymidis[5]. This solution contained (mM): Choline chloride, 138, KCl, 4.7; $CaCl_2$, 2.56; $MgSO_4$, 1.13; Tris (pH 7.2), 5; osmolarity, 300 mosmol/l. The spermatozoa were diluted with the same buffer to a final concentration of 43×10^6 per ml and incubated at 35 °C. This represented the stock suspension from which aliquots (10 µl) were taken up into assay solutions (420 µl) containing normal (138 mM Na^+) or different concentrations of sodium (0 to 143 mM). The ionic compositions of these solution were the same as that of the sodium-free tris buffer except that sodium chloride and choline chloride were present in different proportions

to achieve an osmolarity of 300 mosmol/l. Bovine serum albumin (BSA) (2 mg/ml) and glucose (2.5 mg/ml) were present in all assay solutions, which had a sperm concentration of 1×10^6 per ml. The spermatozoa were incubated in these solutions at 35 °C for 3 h. Sperm motility was measured by the photographic tracking method and expressed as the forward motility index (FMI)[9].

At the end of the 3 h incubation period, the spermatozoa were washed in the normal Na^+ containing solution and incubated in the same solution for 30 min. Forward motility was measured during this recovery period.

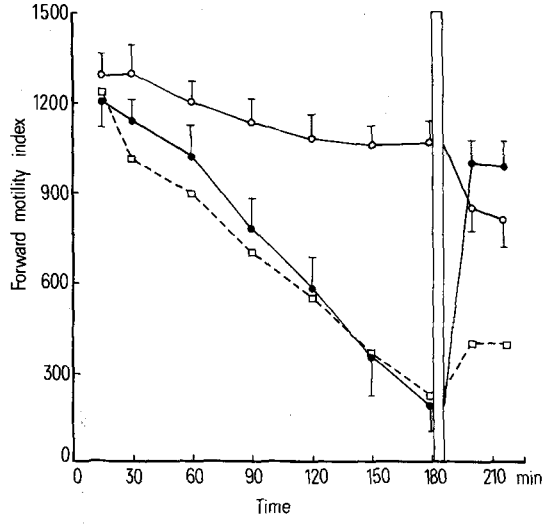

Fig. 1. Effect of complete Na^+ removal (closed circles) on the forward motility of rat caudal epididymal sperm. The open circles show the control. Forward motility was expressed as the forward motility index[9]. Each point shows the mean ± SE of 4 experiments. In 2 experiments, spermatozoa were suspended in a normal Na^+ solution (138 mM) containing 3×10^{-4} M amiloride (squares). At the column, spermatozoa were washed and incubated in the Na^+ containing solution.

Amiloride was added to the normal Na^+ containing solutions to achieve concentrations of 10^{-5} to 3×10^{-3} M. Spermatozoa were incubated in these solutions for 3 h. The FMI was measured at the end of this period.

Results. The effect of complete removal of sodium ions on the forward motility of the rat caudal spermatozoa is shown in figure 1. Spermatozoa incubated in the normal Na^+ (138 mM) solution maintained their motility. In contrast, those incubated in the Na^+-free solution showed a progressive fall in motility and at the end of 3 h the FMI was almost abolished. Upon washing the spermatozoa in the normal Na^+ solution, the forward motility was completely restored. Similar results were obtained with other Na^+-free solutions in which the sodium ions were replaced by sucrose or tris (results not shown).

The effect of different Na^+ concentrations in maintaining motility is shown in figure 2. Spermatozoa were incubated in different Na^+ solutions for 3 h before the FMI was measured. It was found that the motility of spermatozoa altered with Na^+ concentration in a curvilinear fashion showing saturation at 80 mM Na^+. The concentration maintaining 50% motility was 25 mM Na^+.

Incubation of the sperm with amiloride (10^{-3} M) produced effects similar to that of Na^+ removal (figure 1). The inhibitory effect of amiloride on sperm motility was concentration dependent (figure 3). Maximal effect was obtained at 10^{-3} M with an IC_{50} at about 5×10^{-5} M.

Discussion. These results showed that extracellular sodium ions are required for the full expression of the forward motility of the mature rat epididymal spermatozoa. Incubation of the spermatozoa in a Na^+-free solution caused a progressive loss of motility. This effect was reversible on washing the spermatozoa in a sodium-containing solution. Similar dependence on extracellular Na^+ of bull and chimpanzee sperm has also been reported[6]. The curve relating Na^+ concentrations and motility showed that 50% motility was maintained by a Na^+ concentration of about 25 mM and that maximal motility could be achieved at 80 mM Na^+. Amiloride inhibited sperm motility and the effect was similar to that of sodium ion removal. It is pertinent that Na^+ influx into spermatozoa maintains the

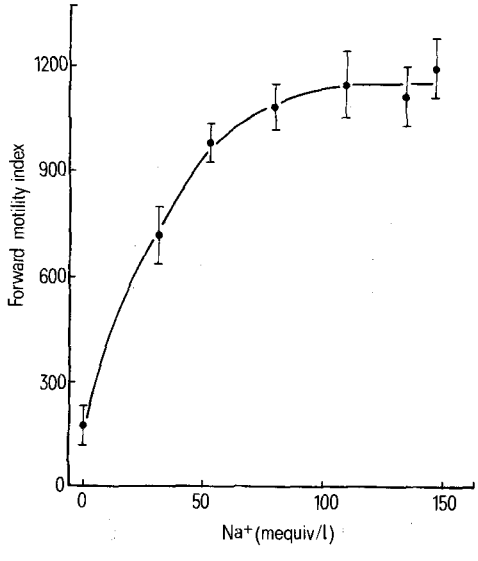

Fig. 2. The effect of different Na^+ concentrations on the maintenance of forward motility of rat caudal epididymal sperm. Forward motility index was measured at the end of 3 h incubation. Each point shows the mean ± SE of 3 experiments.

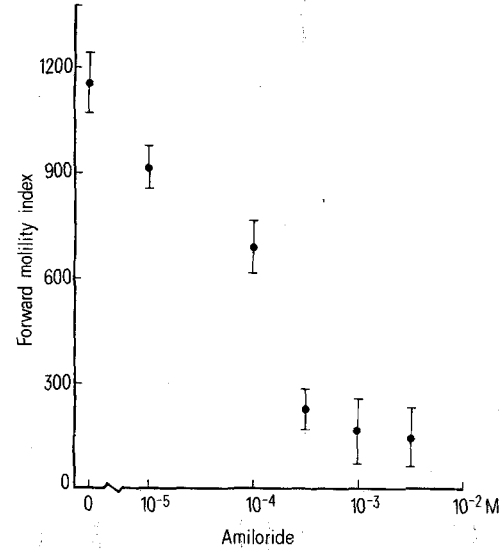

Fig. 3. Log concentration-response curve for amiloride on the forward motility of the rat caudal epididymal sperm. Forward motility index was measured after incubating the sperm with amiloride for 3 h. Each point shows the mean ± SE of 3-6 experiments.

cells in a motile state. Amiloride, which blocks Na^+ influx into cells, may lead to arrest of this process. The dose response curve relating amiloride concentration to sperm motility revealed an IC_{50} of about 5×10^{-5} M. This value is high when compared to those relating the inhibition of amiloride to Na^+ transport in other mammalian epithelia[8,10-12].

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2 The technical assistance of Mr C.M. Li and the gift of amiloride from Merck, Sharp and Dohme are gratefully acknowledged.

3 N. Levine and D.J. Marsh, J. Physiol., Lond. *213*, 557 (1971).
4 T.T. Turner, P.K. Hartmann and S.S. Howards, Fert. Steril. *28*, 191 (1977).
5 P.Y.D. Wong and C.H. Yeung, J. Physiol., Lond. *275*, 13 (1978).
6 A.V. McGrady, L. Nelson and M. Ireland, J. Reprod. Fert. *40*, 71 (1974).
7 J.C. Wallace and R.G. Wales, J. Reprod. Fert. *8*, 187 (1964).
8 A.W. Cuthbert, in: Drugs and transport processes, p. 173. Ed. B.A. Callingham. Macmillan, London 1974.
9 T.S. Acott and D.D. Hoskins, J. biol. Chem. *253*, 6744 (1978).
10 P.Y.D. Wong and C.H. Yeung, Br. J. Pharmac. *58*, 529 (1976).
11 J.P. Guignard and G. Peters, Eur. J. Pharmac. *10*, 255 (1970).
12 L.H. Schneyer, Am. J. Physiol. *219*, 1050 (1970).

Functional absence of brain photoreceptors mediating entrainment of circadian rhythms in the adult rat

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Summary. The photic energy penetrating into the brain was increased in adult rats sustaining craniotomies sealed with transparent plastic. After blinding, these animals failed to entrain their circadian food intake rhythm to light-dark cycles. Short pulses of light did not phase-shift the freerunning rhythm. We conclude that adult rats lack brain photoreceptors mediating entrainment of circadian rhythms.

In submammalian vertebrates photic entrainment of circadian rhythms can be mediated by an extraretinal photoreceptive system in the brain[1]. In mammals, on the other hand, blinding results in freerunning rhythms suggesting that entrainment is exclusively mediated by the retina[2]. However, substantial amounts of light penetrate into the brains of mammals[3]. Moreover, light impinging directly on hypothalamic cells can evoke photo-neuro-endocrine reflexes in blind adult rats[4]. It has also been shown that the pineal serotonine-N-acetyltransferase activity rhythm of blinded neonatal rats is entrained to the light-dark cycle. The photoreceptors for this entrainment lie within the animal's brain[5]. Since pineal photoreceptors have not been demonstrated in the rat[6], and, accordingly, pinealectomy in this species does not interfere with entrainment[7] these findings suggest a functional role for hypothalamic photoreceptors mediating entrainment in immature rats. This led us to investigate whether the circadian food intake rhythm of blinded adult rats can be entrained if the amount of light entering the brain is increased.

Materials and methods. In 20 adult male rats blinded by binocular enucleation the circadian rhythm of food intake was continuously recorded by monitoring the number of food approaches in 30-min intervals[8]. Throughout the experiment the animals were exposed to light-dark cycles (L:D 12:12 h; L = 300–400 lx). In 5 additional blind animals a hole was drilled in the skull at or just rostral to the bregma, exposing over 28 mm^2 of brain surface. The holes were sealed with a thin layer of transparent plastic. Thus light could penetrate directly into the brain. At a later stage of the experiment the animals sustaining such craniotomies were exposed to intense 1-h (white) light pulses (1600 $\mu W \cdot cm^{-2}$) at various phases of their circadian cycle. For this purpose they were anaesthetized with Hypnorm (Philips-Duphar, 1 $ml \cdot kg^{-1}$) and placed with the cranial opening in the beam of light. Control craniotomized animals were merely anaesthetized at corresponding phases.

Results and discussion. The 20 animals without craniotomies exhibited freerunning circadian food intake rhythms after binocular enucleation with periods exceeding 24 h (mean: 24.27 h; range: 24.09–24.47 h). Exposure to the L:D 12:12 lighting cycle therefore did not result in entrainment. Moreover, for none of the animals was evidence for passing synchronization obtained[9]. These findings are illustrated for a blinded rat in figure 1, B. Similarly, the rhythms of the craniotomized rats were freerunning with comparable periods (mean: 24.21 h; range: 24.07–24.38 h, e.g., figure 1, A). To further investigate the possible presence of an extraretinal photoreceptive system the brains of the craniotomized animals were illuminated with single 1-h light pulses of high intensity (1600 $\mu W \cdot cm^{-2}$). In sighted rats kept under conditions of constant illumination such pulses are known to result in phase shifts of the freerunning rhythm, the direction and magnitude of which systematically depend on the phase of the circadian cycle at which the light pulse is delivered[10]. Figure 2 summarizes the results of

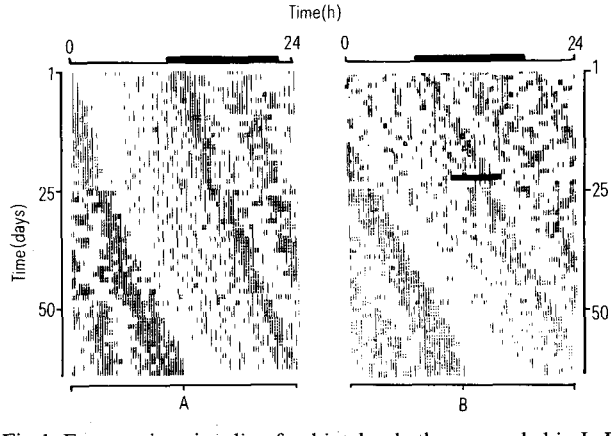

Fig. 1. Freerunning circadian food intake rhythms recorded in L:D 12:12 (as indicated above the records) for *A* a blinded craniotomized and *B* a normal blinded rat. The dark rectangle on day 23 in record B indicates the time and duration of control Hypnorm anaesthesia.