and nitrophenol, too, but not by salicylic acid and the compound HOE 17,879 (table). Aminopyrine N-demethylase which is inhibited by the compound HOE 17,879[21] is not inhibited by furosemide, in concentrations up to 10 mM.

Discussion. The fact that furosemide inhibits microsomal uridinediphosphate glucuronyltransferase, is to be seen in connection with the fact that it is glucuronidized by this enzyme itself[10]. So one would expect a competitive type of inhibition, but in fact the inhibition was shown to be a non-competitive one. The authors are reluctant to draw too many conclusions from this unexpected finding, because the type of inhibition needs to be studied with solubilized and purified enzyme preparations, rather than in crude microsomes, to investigate in detail the kind of interaction between furosemide and the conjugating enzyme. In contrast to the microsomal enzyme inducer, HOE 17,879 (2-hydroxy-2-ethylbutyryl-N,N-diethylamide) which inhibits drug-metabolizing, cytochrome P-450-dependent mixed-function oxidases of liver microsomes, both in vivo[20] and in vitro[21] but has no effect on glucuronyltransferase (see Beyhl[21], cf. table), furosemide does not influence aminopyrine N-demethylase in vitro which agrees well with the in vivo findings of Muschaweck and Beyhl (unpublished) namely that aminopyrine N-demethylase activity is not affected by furosemide, neither in acute nor in chronic treatment with this drug. From our studies with furosemide and some other diuretics as well as with probenecid, we have got the impression that the carboxyl group must be present for inhibition of the glucuronyltransferase, in this series of compounds.

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Autoradiographic evidence for binding of ^{3}H-flunitrazepam (Rohypnol®) to melanin granules in the cat eye

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Summary. This autoradiographic study revealed after an i.v. injection an accumulation of ^{3}H-flunitrazepam in melanin granules of the pigment epithelium and of the choroid cells. It seems that after a short period, a large proportion of the strongly bound drug is released.

Recently it was demonstrated by whole-body autoradiography (Prof. J. Rieder and Dr R. Heintz, personal communication) that flunitrazepam (Rohypnol®) shows an affinity to melanin-containing tissues, like the retinotoxic drug chloroquine[2-4]. After a single injection in pregnant mice, ^{14}C-marked flunitrazepam was visible in the uvea of pigmented offspring even 100 days after the injection. The present study examined the localization of ^{3}H-flunitrazepam in the tissues of the cat eye at an ultrastructural level, in connection with a toxicological study[5] of chloroquine and flunitrazepam.

^{3}H-flunitrazepam (labeled at position 9; sp. act. = 60.67 mCi/mg)[6] was used at a concentration of 2 mg/ml in the solvent as for human injection. 2 male cats of approximately 2.5 kg received 1 mg/kg via the saphenous vein in 20 min with an infusion pump. For this application the cats were anaesthetized with an intramuscular injection of 14 mg/kg Vetalar®. 2 other male cats were treated from Monday to Friday the 1st week and from Monday to Thursday the 2nd week with 1 mg/kg (total 9 injections) ^{3}H-flunitrazepam daily. The drug was injected into the left jugular vein of non-anaesthetized cats through a mounted Vercath catheter within 1–2 min each. After 1 and 14 days, respectively, the eyes were carefully dissected from narcotized cats (35 mg/kg Nembutal, i.p.), and cleaned of adherent tissue. An incision into the front of the cornea was made with a scalpel, and the eye fixed in the half-concentrated Karnovsky solution[7] for 30 min under rotation at room temperature. The eye was divided perpendicular to the optical axis (just behind the ciliary body) with a razor-blade. The fundus, separated from the vitreous body, was cut into small (2 × 3 mm) pieces (tapetum lucidum and tapetum nigrum), and fixed for a further 90 min in the same fixative, but at 0–4 °C. After rinsing in 0.1 M sodium cacodylate + 7% sucrose, buffered at pH 7.4, the tissue was fixed for 60 min in 2% osmium tetroxide, contrasted in 0.1% uranyl acetate for 30 min, and dehydrated with ethanol/propylene oxide and embedded in Epon.

Ultrathin sections through the retina and adjcent tissue were mounted on silver grids, and coated with Ilford L4 emulsion by the loop method[8]. After an exposure time of between 4 and 28 days at 4 °C, the emulsion was developed with Kodak D 19 for 5 min at 20 °C, and fixed for 5 min with Kodak rapid fixer. The sections were stained with lead citrate and examined with a Philips EM 300 electron microscope.

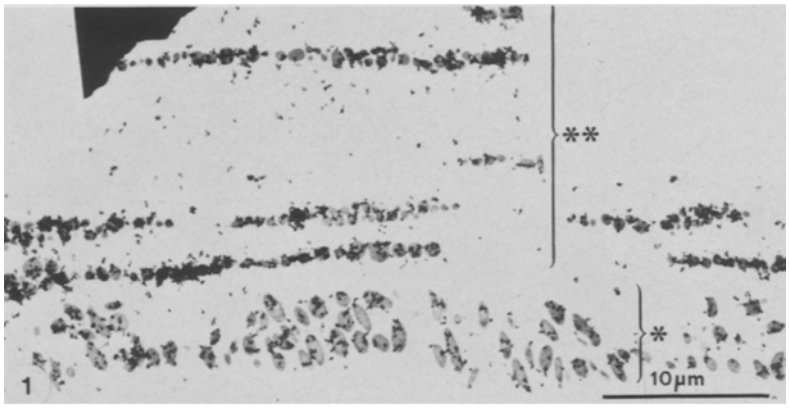

Fig. 1. Autoradiographic localization of ^{3}H-flunitrazepam in the melanin-containing structures of the cat eye. Chronic administration (group 3); exposure time = 28 days. A marked localization is visible in the melanin granules in the pigment epithelium (*) and in the choroid cells (**).

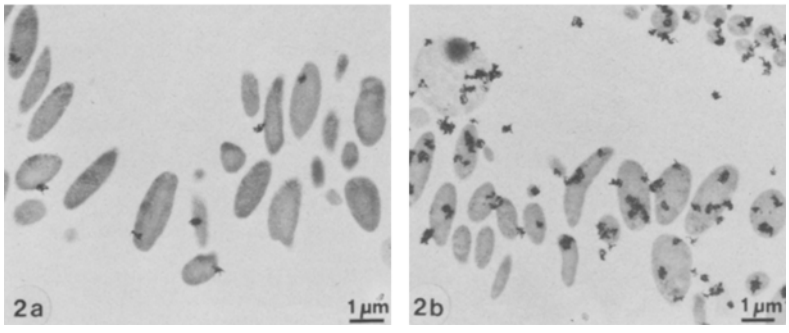

Fig. 2. Autoradiographic localization of ^{3}H-flunitrazepam in melanin granules in the pigment epithelium of the cat. *a* Acute administration (group 2); exposure time = 8 days. Few silver grains are localized on the melanin granules of the pigment epithelium. *b* Chronic administration (group 3); exposure time = 8 days. On average, approximately 3 silver grains are localized on each melanin granule of the pigment epithelium.

In all treated cats, silver grains were selectively distributed in the melanin granules of the pigment epithelium and of the choroid (figure 1). Treatment of 1 cat with 1 mg/kg ^{3}H-flunitrazepam, and fixing of the tissues 1 day later (group 1), led to a localization of silver grains in melanin granules after an exposure time of 4 days. When tissues were fixed 14 days after a single injection given to another cat (group 2), an exposure time of 14 days was necessary for a similar density of silver grains in melanin granules (figure 2, a). In tissues fixed 1 day after the last of 9 injections, administered within 13 days (group 3), a marked increase in the number of silver grains in the melanin granules was observed in both cats after an exposure time of 8 days (figure 2, b).

A semi-quantitative determination of the number of silver grains per melanin granule in the pigment epithelium was made for the 3 different treatments (groups 1-3) at exposure times of 4, 8, 14 and 28 days (figure 3). For these measurements, all sections were prepared at the same time, using the same emulsion. The 9-fold dosage led only to a double, not to a 9-fold increase in the number of silver grains per melanin granule. On the other hand, between 1 and 14 days after a single injection (groups 1-2), approximately 50% of the activity, i.e. of the bound ^{3}H-flunitrazepam, was released.

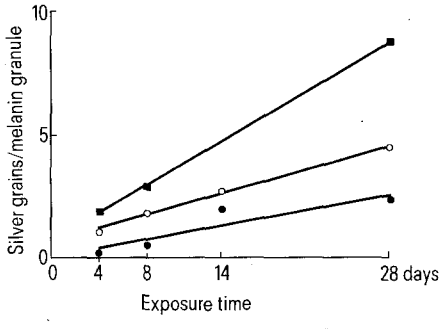

Fig. 3. A semi-quantitative autoradiographic determination of ^{3}H-flunitrazepam in melanin granules of the cat pigment epithelium over the tapetum nigrum. ○ (group 1, n = 1); ● (group 2, n = 1); ■ (group 3, n = 2).

The affinity of chloroquine to melanin-containing structures[2-4] was postulated as a causal factor in retinal damage[9]. In spite of the affinity of flunitrazepam to the melanin granules, no effect on the structure of the pigment epithelium or the retina in cats or mice was observed, even after 2000 times (mice) or 130 times (cats) the effective human daily dose. The semi-quantitative determination of ^{3}H-flunitrazepam bound to melanin granules (by counting the number of silber grains per melanin granule) allowed the assumption that, after injection, the flunitrazepam is strongly bound to the melanin granules, but that after a short period (14 days) a large proportion (approximately 50%) is released. Furthermore, repeated injections of flunitrazepam did not lead to a proportionally increased accumulation. It seems that the melanin granules become saturated with flunitrazepam. Therefore, long-term treatment may not cause a much higher concentration of flunitrazepam in the melanin granules to be reached.

Several authors have shown, in toxicological studies with flunitrazepam[5] and chloroquine[4,5,10-12] that the retinotoxicity of chloroquine is not a function of its affinity to melanin. The chloroquine toxicity to the retina was not only demonstrated in pigmented, but also in albino animals[10-13]. The most notable structural change was observed in the area of the cat retina with a low melanin content, namely in the ganglion cells and in the tapetal cells[5]. On the other hand, in chloroquine-treated cats the pigment epithelium lacking melanin granules (over the tapetum lucidum) was more affected than that containing melanin granules (over the tapetum nigrum). It seems therefore that the retinotoxic effect of chloroquine is related not to its affinity to melanin but possibly to its neurotoxicity[14]. There is no evidence to class a substance with an affinity to melanin-containing tissues as potentially harmful to vision.

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Ureteral contractions induced by rat urine in vitro: probable involvement of renal kallikrein

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Summary. Rat urine, even at a 1:10 final dilution in Tyrode's solution, stimulates contraction of the ureteral musculature in vitro. This effect can be ascribed to the presence of kallikrein or a kallikrein-like enzyme in urine. Isometric contractions of ureters were prevented by previous addition of aprotinin to the organ bath. Urine also lost its activity after inactivation of enzymes by heat or acid treatment.

Renal kallikrein, an enzyme clearly different from plasma kallikrein, has been localized in the distal nephron[2,3]. At this site it is probably released into the tubular fluid, and then excreted in the urine[4,5]. The physiological role of this enzyme is still unknown, although it has been suggested that it may be involved in the control of sodium excretion[6,7], in the renal response to mineralocorticoid hormones[8,9] or in the control of renal blood flow[10]. Renal kallikrein could also have a function beyond this organ. Kallikrein stimulates contraction of the isolated dog intestine and of the isolated rat uterus in oestrous[11-14]. This activity has been used to measure kallikrein biologically[5,15]. The urinary tract has a common embryologic origin with the distal nephron, therefore, it appeared to be of interest to investigate whether rat urine, through its content of kallikrein, could also stimulate the ureteral smooth muscle in vitro.

Methods. Ureters were obtained from rats sacrificed by a blow on the head. Both ureteral ends were ligated and the organs immersed, with 0.1 g tension, in 12–15 ml Tyrode's solution (NaCl 128 mM, KCl 4.7 mM, $NaHCO_3$ 11.9 mM, NaH_2PO_4 1.2 mM, $MgCl_2$ 1.2 mM, $CaCl_2$ 2.5 mM, glucose 10 mM) kept at 37 C in a glass bath and constantly flushed with 5% CO_2 and 95% O_2. Isometric contractions were amplified and registered by means of a Heathkit EU20B recorder.

24-h-urine samples were collected from rats (n = 10) placed in metabolic cages and fed normal rat chow and with free access to drinking water. Urine was kept frozen until used. Urine was added to the isolated muscle bath immediately after thawing or after dialysis against 2 changes of isotonic saline chloride or Tyrode's solution (volume ratio 1:200) at 4 C for 48 h. Urinary enzymes were inactivated by immersing the dialyzed aliquots in a boiling water bath for 60 min or by the addition of one volume of 10% trichloroacetic acid (TCA). TCA-precipitated urine samples were neutralized with NaOH prior to addition to the organ bath. After 2–4-min contact time the preparation was rinsed with fresh Tyrode's solution.

Results. Rat urine induced an ureteral contraction of a moderate tonic character with superimposed clonic episodes (figure 1). The contractions of the isolated ureters continued until rinsing (figure 3). In some preparations repetitive addition of urine resulted in a disappearance of effect. The type of contraction elicited was identical to that evoked by bradykinin. Prolonged dialysis of urine aliquots did not affect contractile activity which, however, disappeared after enzyme inactivation by boiling (figure 1) or acid treatment of urine (figure 2). The stimulating activity of rat urine could be totally suppressed by a serine protease inhibitor (aprotinin, 1000 kIU/ml) added to the organ bath (figure 3).

Discussion. The effect of bradykinin on the ureteral musculature of dogs and rats in vivo was described several years ago[16]. The influence of bradykinin on ureteral musculature in vitro, has not yet been reported , to our knowledge.

The present study indicates that rat urine at a 1:10 final dilution induces contractions of isolated ureters. The active principle is not dialysable and can be inhibited by heat or acid denaturation, and blocked by the previous addition of aprotinin to the organ bath. The response could be markedly reduced, albeit not inhibited, by the addition of bradykinin-binding antibodies to the bath. This could be due to a greater association constant for bradykinin with receptors than with antibodies, or to an incapacity of the antibodies to compete against bradykinin to reach the site at which kallikrein releases the biologically active kinins. It may be concluded that the observed ureteral stimulation by urine depends on its kallikrein content or on a kallikrein-like enzyme, which would release kinins from kininogen present in the ureters. The amount of kininogen available in the isolated ureters (weighing 5–10 mg) is undoubtedly very small. If kallikrein stimulation of the ureter is mediated through kinin release from kininogen, the disappearance of response in some ureters could be due to consumption of the available kininogen. Since urine was added to the serosal side of the ureters, the present experiments suggest but do not prove that the kallikrein-kinin system modulates ureteral peristalsis in vivo.