and they generally corresponded to the graphical readings. Thus, significance limits are a useful tool for graphical data representations, since they indicate the statistical uncertainty of a value, and also allow approximate statistical comparisons between two or more data sets.

1 Velleman, P. F., and Hoaglin, D. C., Applications, Basics, and Computing of Exploratory Data Analysis. Duxbury Press, Boston, MA, 1981.

2 Three different sets of values with an a priori mean of 0 and standard deviation of 1 were generated. The first set was multiplied with 0.6 (thereby reducing its a priori standard deviation by the same factor) and incremented by 3.78; the second set was just incremented by 4.05 and the third set by 4.25. The a posteriori means and standard deviations are listed in fig. 1. F-tests for comparing the variances resulted in following error probabilities: set 1 vs set 2: $p = 0.078$; set 1 vs set 3: $p = 0.009$; set 2 vs set 3: $p = 0.164$.

3 Welch, B. L., Biometrika *36* (1949) 293. This procedure for unequal variances converges to the one for equal variances when the standard deviations become equal. Therefore, it has been used for all non-graphical t-tests.

4 Andrews, H. P., Snee, R. S., and Sarner, M. H., Am. Statistn *34* (1980) 195; Hochberg, Y., Weiss, G., Hart, S., J. Am. statist. Ass. *77* (1982) 767; Godfrey, K., N. Engl. J. Med. *313* (1985) 1450.

5 For ease of implementation and for clarity (context independence), only information from the considered individual data set should be included in the definition of an uncertainty measure. Therefore, unequal variances and sample-sizes, as well as comparison multiplicity were not taken into account for the definition of the significance limits. It is, however, important that the user of significance limits becomes aware of the imprecisions which may result from these simplifications.

6 See for example Elsner, J., Looser, R., and Zbinden, G., Neurobehav. Toxic. Terat. *1*, suppl. 1 (1979) 163; Schlatter, J., Elsner, J., and Zbinden, G., Neurobehav. Toxic. Terat. *5* (1983) 413; Looser, R., Elsner, J., and Zbinden, G., Psychopharmacology *84* (1984) 323; Elsner, J., Neurobehav. Toxic. Terat. *8* (1986) 573; Elsner, J., Fellmann, Ch., and Zbinden, G., Neurobehav. Toxic. Terat. *10* (1988) 3; Elsner, J., Hodel, B., Suter, K. E., Oelke, D., Ulbrich, B., Schreiner, G., Cuomo, V., Cagiano, R., Rosengren, L. E., Karlsson, J. E., and Haglid, K., Neurobehav. Toxic. Terat. *10* (1988) 155; Elsner, J., Alder, S., and Zbinden, G., Psychopharmacology *96* (1988) 194; Tannhauser, S. L., Elsner, J., Tannhauser, M., Barros, H. M. T., and Tannhauser, M. A., Brazilian J. med. biol. Res. *22* (1989) 213; Balduini, W., Lombardelli, G., Peruzzi, G., Cattabeni, F., and Elsner, J., Neurobehav. Toxic. Terat. *11* (1989) 339; Elsner, J., Alder, S., and Fellmann, Ch., Neurobehav. Toxic. Terat. *12* (1989) 7.

7 For the formulae of the confidence and tolerance limits see e.g. Wissenschaftliche Tabellen Geigy, Vol. 3, p. 206–207. Ciba-Geigy AG, Basel 1980.

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Aluminium in repair membranes and Al, Ca and P_i in the haemolymph of Al-injected shell-repairing snails (*Helix pomatia* L.)

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Summary. In $AlCl_3$-injected shell-repairing snails, *Helix pomatia* L., the Al-associated decrease of the weights of the shell-repair membranes was unrelated to the Al-concentration in the membranes. In the haemolymph the concentration of Al was related to the dose of injected Al, while the concentration of Ca was increased by the highest Al-dose only. No phosphate was detected in either controls or Al-injected snails. It is concluded that Al inhibits the growth of the $CaCO_3$-crystals by mechanisms other than incorporation in, or adsorption to, the crystals.
Key words. Aluminium; snail; shell-repair; calcium; phosphate.

Aluminium (Al) has been shown in laboratory investigations to affect the process of shell-repair in the snail *Helix pomatia* L. Both the weights and the relative calcium concentration in the shell-repair membranes are reduced in snails injected with $AlCl_3$ [1]. These effects resemble those recorded in the skeletal bones both from patients suffering from the Al-associated syndrome of dialysis osteomalacia and from Al-treated experimental animals [2–4]. Aluminium has furthermore been demonstrated to inhibit the calcification of demineralized shell-repair membranes in vitro, and to reduce the formation of calcium carbonate ($CaCO_3$), the mineral found in the shell of the snail, in a pure physical-chemical system [5]. Similar effects of Al have also been reported concerning the in vitro formation of calcium phosphate [6, 7]. Aluminium possibly interferes with crystal growth by adsorbing to the crystal surfaces. The presence of Al in the lower-weight repair membranes of the Al-injected snails thus indicates a direct effect of Al on the growth of the $CaCO_3$-crystals, while the absence points towards an indirect mechanism.

Aluminium is also known to interfere with phosphate metabolism [3, 8]. Energy-requiring processes like the production of the organic matrix of the repair membrane and the transport of ions across the mantle epithelium (see Watabe [9] for a review of the shell-repair process) may thus be affected. A disturbed phosphorus metabolism in the Al-treated snails might be detected as a reduced concentration of inorganic phosphate (P_i) in the haemolymph.

The induction of acidosis, e.g. by the injection of an acidic solution, increases the Ca-concentration in the haemolymph [10]. Elevated Ca-concentrations in animals injected with $AlCl_3$, which is an acidic solution, thus

indicate that acidosis has been induced and still persists at the time of blood sampling.

Finally, the theoretical Al-concentration in the haemolymph immediately following the injections[1], compared with the actual concentrations found 4 days later at the time of blood sampling, would give information concerning the fate of the injected Al.

It was thus decided to investigate the effects of Al-injections and shell-repair on the concentration of Al in the repair membranes and the concentrations of Al, Ca and P_i in the haemolymph.

Materials and methods

Snails, *Helix pomatia* L., were collected and kept in the laboratory as previously described[1]. The animals, all adults with a mean weight of approximately 25 g, were divided into 3 groups (A, B and C) of 8 animals each. On day 1 of the experiment shell-damage was inflicted; this procedure and the recording of the weights of the resulting shell-repair membranes were performed according to Reineskog[1]. The membranes were removed on day 2 (these membranes being discarded), on day 3, and on day 9, following repeated damage on day 8. On days 4 and 5 the snails received sham-injections (group A), or injections (10 µl/g b. wt) of 10 mM $AlCl_3$ (group B), or 100 mM $AlCl_3$ (group C). The pH of the two Al-containing solutions were adjusted to 3.2 and the injections were made as previously described[1].

Following the determination of the weights of the dried repair membranes they were digested according to Uhrberg[11]. Aluminium was then determined by graphite furnace atomic absorption spectrophotometry. Using a Varian AA-6 instrument equipped with a Perkin-Elmer HGA-76 graphite furnace and a deuterium background correction system, the method of standard addition[12] was employed.

On day 9, after the removal of the repair membranes, approximately 1 ml of haemolymph was collected from each snail with a needle and syringe inserted into the 'lung-vein', pointing towards the heart. Aluminium in the haemolymph was then analyzed by the same procedure as described above for the repair membranes with the exception that the haemolymph, following dilution 1:5 with ethanol, was made slightly acidic and injected directly in the graphite furnace without further digestion. Calcium was determined by flame photometry using an Eppendorf flame photometer (Eppendorf Gerätebau Netheler & Hinz GmbH, Hamburg, FRG); the procedure described by the manufacturer for the determination of Ca in serum being adopted. Inorganic phosphate was determined spectrophotometrically by a modified version of the method described by Taussky and Shorr[13] for the determination of inorganic phosphorus in serum. To 0.4 ml of haemolymph 1.5 ml of 730 mM trichloroacetic acid was added. Thereafter 1.5 ml freshly prepared colour reagent (0.93 mM ammoniumheptamolybdate and 33 mM ferrous sulfate dissolved in 660 mM sulfuric acid) was added. The mixture was centrifuged at 1000 × g for 10 min and the absorption of the supernatant read at 700 nm 1 h after the addition of the colour reagent. A standard curve, ranging from 25 to 1000 µM inorganic phosphate, was prepared using the same analytical procedure.

All chemicals used were of analytical grade, or as $AlCl_3$ (Fisher Scientific Co., Fair Lawn, New Jersey, USA; lot No. 711755), of certified grade.

The Wilcoxon Rank sum-test ($p < 0.05$ being considered significant) was employed for statistical evaluation of the experimental data.

Results and discussion

On day 3, before the Al-injections, all 3 groups (A, B and C) had mean membrane weights statistically indistinguishable from each other. On day 9 the weights of the membranes from the Al-treated groups (B and C) were significantly lower than those recorded for the sham-injected group (A). The Al-concentrations in the repair membranes did not differ significantly between the 3 groups either before or after the Al-injections (table). One snail from group C died before day 9 while another one from this group failed to repair the damaged shell. The lack of correlation between the Al-contents and the weights of the repair membranes renders the proposal of a direct inhibitory effect of Al on the formation of the $CaCO_3$-crystals less plausible. An adsorption of Al to the crystal surfaces, or an incorporation of Al instead of Ca

Effects of sham-injections (A) and injections (10 µl/g body weight) of 10 or 100 mM $AlCl_3$ (B and C, respectively) on the weights and the Al-concentrations in shell-repair membranes from shell-repairing snails, *Helix pomatia* L. Also shown are effects on Al, Ca and inorganic phosphate (P_i) in the haemolymph.

Treatment	Shell-repair membranes weights (mg) day 3	day 9	Al-contents on day 9 (µg/g)	Haemolymph Al (µM) (concentrations on day 9)	Ca (mM)	P_i
Sham (group A)	3.8 (2.2–4.7)	5.5 (3.7–6.6)	1.2 (0.70–4.0)	0.28 (0.15–9.82)	2.3 (1.6–3.0)	n.d.
10 mM $AlCl_3$ (group B)	3.7 (2.3–4.9)	3.7* (2.2–4.5)	2.1 (1.0–7.9)	100* (89–180)	1.9 (1.2–2.9)	n.d.
100 mM $AlCl_3$ (group C)	4.1 (3.0–4.7)	3.2* (1.9–4.1)	1.7 (0.50–5.4)	880** (700–1200)	3.4** (2.2–3.9)	n.d.

Values are medians and range (n = 8 except for the day-9 values of group C where n = 6); * significantly different from group A (Wilcoxon Rank-sum test, $p < 0.05$); ** significantly different from groups A and B (Wilcoxon Rank-sum test, $p < 0.05$); n.d., none detected.

in the crystals, would most likely have been detected as an increased Al-concentration. Instead, the presence of equally low Al-concentrations in the membranes from both controls and Al-injected snails indicates that no or only very small amounts of Al are transported across the mantle epithelium. Aluminium thus appears to inhibit the repair at an earlier stage than the actual crystal formation.

The Al-concentration in the haemolymph of the Al-injected snails was significantly higher than that recorded in the control group. The group injected with 100 mM $AlCl_3$ had an increased concentration also vis-à-vis the group receiving 10 mM $AlCl_3$ (table). Comparing the Al-concentration in the haemolymph of the snails injected with 10 mM $AlCl_3$ with the theoretical Al-concentration following such injections (454 μM [1]), approximately 80% of the Al is calculated to have been eliminated from the haemolymph. Also in the animals injected with 100 mM $AlCl_3$ roughly the same percentual amount of Al has been eliminated; the ratio between the Al injected and the Al present in the haemolymph being the same for the two Al-doses. Also, the distribution of Al between the haemolymph and the other compartments of the animal appears to be the same regardless of the Al-dose. Neither the capacity to store nor to excrete the injected Al seems to have been exceeded. However, the higher the Al-dose the higher the Al-concentration in the haemolymph and the possibility of interactions with the normal metabolism.

Regarding the Ca-concentration in the haemolymph, group C showed a significant increase compared with both groups A and B (table). The snails of group C (treated with 100 mM $AlCl_3$) thus appears to be in an acidotic state even several days after the acidic injections. The Ca-stores activated by acidosis are reported to be the same as those used for shell-repair [14]. However, as previously shown [1], the process of shell-repair is unaffected by the injection of acid. The present finding of reduced membrane weights in snails injected with 10 as well as 100 mM $AlCl_3$, but of acidosis only in the latter, also shows the lack of association between acidosis and reduced membrane weights.

No inorganic phosphate was detected in the haemolymph of any group. The concentration of P_i is thus below 25 μM, the lowest concentration of standards used. At pH 7.80, the normal pH of the haemolymph of *H. pomatia* [15], the presence of Al limits the concentration of free inorganic phosphate to only 20 pM [1]. In the present study the phosphate concentration in the haemolymph was found to be lower than previously reported (50–160 μM), as reviewed by Burton [16]. However, as the phosphate concentration most probably exceeds 20 pM, $AlPO_4$ will precipitate. The phosphorus metabolism, and the energy-requiring repair process, may thus be disturbed.

In conclusion, the Al-associated inhibition of the repair system is probably not the result of a direct effect of Al on the physical-chemical process of the $CaCO_3$ crystal formation. However, by interacting with the EDTA-soluble regulatory molecules that control initiation and termination of the calcification [17], Al may indirectly affect crystal formation and growth. Other possible targets of Al toxicity are the movement of calcium and carbonate over the mantle epithelium, or the production of the organic matrix of the repair membrane, performed by the mantle.

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