The Effect of Ambient Calcium on Prolactin Cell Activity and Plasma Electrolytes in Sarotherodon mossambicus (Tilapia mossambica)

S. E. Wendelaar Bonga and J. C. A. van der Meij

Department of Zoology, University of Nijmegen, Nijmegen, The Netherlands

Accepted October 10, 1979

The low prolactin cell activity in seawater fish is related to the high calcium and magnesium concentrations in seawater, not to its high osmolarity or high sodium content. After transfer of freshwater fish to seawater, prolactin cell activity was markedly reduced. A similar reduction occurred in freshwater fish after increasing the ionic calcium concentration to that of seawater. Magnesium ions, although considerably less effective than calcium in the same concentration, had a similar effect. In seawater fish prolactin cells were stimulated by reduction of the ambient calcium and magnesium concentrations. Data on blood plasma composition do not support the supposition that the effect of ambient calcium on prolactin secretion is mediated by changes in plasma osmolarity, plasma sodium, or plasma calcium levels. Prolactin is known to reduce the permeability of the fish integument for ions and water. Similar effects have been reported for ambient calcium and magnesium ions. The present results support the hypothesis that prolactin compensates for the effects of low ambient calcium and magnesium levels on water and ion permeability of the skin and gills, in hypotonic as well as hypertonic conditions.

When seawater-adapted, euryhaline, teleost fish are transferred to fresh water, prolactin cell activity is enhanced in many species. The activation of the prolactin cells has been related to the low osmolarity and low sodium content of fresh water, and prolactin has therefore been implicated in the endocrine control of adaptation to a hyposmotic environment (Ensor and Ball, 1972; Schreibman et al., 1973; Nagahama et al., 1973). The effect of external osmolarity has been assumed to be mediated by changes in plasma sodium or osmolarity. This hypothesis is supported by in vitro studies. In organ culture, a reduction of the osmolarity of the culture fluid has been reported to stimulate prolactin secretion (Baker and Ingleton, 1975; Nagahama et al., 1975).

Experiments on the stickleback Gasterosteus aculeatus have shown, however, that in this species prolactin cell activity is not primarily related to environmental osmolarity. The concentrations of calcium and, to a lesser extent, magnesium ions proved to be the main environmental factors controlling prolactin secretion in freshwater and seawater fish. Exposure of fish to high external calcium concentrations led to a reduction of prolactin cell activity, while an increase was observed when the external calcium levels were reduced. No relationship was found between prolactin cell activity and plasma osmolarity or sodium content (Wendelaar Bonga, 1978a, b).

To establish whether such relations are found in other euryhaline fish, we studied a taxonomically unrelated species, the cichlid Sarotherodon mossambicus. This species was selected since the mode of control of its prolactin cells has been the object of several studies, in vivo as well as in vitro. These studies led to the conclusion that external and internal osmolarities are the main factors involved (Nagahama et al., 1973; 1975). We studied prolactin cell activity and several blood parameters in fish exposed to media varying in osmolarity, sodium content, and calcium concentra-
tion. Prolactin cell activity was estimated by morphometrical techniques at light and electron microscope level. Its relation with blood composition was analyzed by measuring plasma osmolarity and plasma sodium, chloride, and calcium levels.

**MATERIALS AND METHODS**

The animals used were sexually mature male *Sarotherodon mossambicus* of about 12-cm body length, ranging in body weight from 15 to 20 g. They were obtained from laboratory stock and represented the second and third generations of fish caught in their natural habitat in Tanzania. They were kept in 100-liter freshwater aquaria at 25° and a 12-hr photoperiod (8 AM–8 PM). The water was continuously recirculated through gravel and charcoal filters. They were fed daily throughout the experiments with Tetramin tropical fish food and twice a week with minced beef heart.

The fish were exposed for 4 weeks to one of the following solutions.

(a) **Artificial fresh water.** Demineralized water containing (in mmol/liter): NaCl, 2.10; Na₂SO₄, 0.45; KCl, 0.06; CaCl₂, 0.80; MgCl₂, 0.20; pH 7.6.

(b) **Sodium-enriched fresh water.** As under (a), but with 490 mmol/liter NaCl. The NaCl concentration of artificial fresh water was daily increased and the final concentration of 490 mmol/liter was reached at Day 6 of the experimental period.

(c) **Calcium-enriched fresh water.** As under (a), but containing 10.2 mmol/liter CaCl₂. The CaCl₂ concentration of artificial fresh water was daily increased and the final concentration of 10.2 mmol/liter was reached at Day 6 of the experimental period.

(d) **Artificial seawater.** Demineralized water containing (in mmol/liter): NaCl, 30.0; KCl, 9.7; CaCl₂, 10.2; MgCl₂, 56.0; Na₂SO₄, 28.2; NaHCO₃, 2.3. Fish kept for at least 4 weeks in natural seawater were transferred to this solution at the start of the experimental period. The solution is similar to regular seawater as far as the concentrations of the main ionic components are concerned.

(e) **Low-calcium, low-magnesium seawater.** As under (d), but with 0.8 mmol/liter CaCl₂ and 5.6 mmol/liter MgCl₂. Fish kept for at least 4 weeks in natural seawater were transferred to artificial seawater. The calcium and magnesium concentrations were decreased daily and the final concentrations were reached at Day 6 of the experimental period.

The pituitary glands of the fish from groups a–e were studied at light and electron microscope levels. Additional light microscope observations were made on fish exposed for 4 weeks (including an adaptation period of 6 days) to fresh water containing 5.6 or 56 mmol/liter MgCl₂, and to low-calcium seawater (Ca²⁺: 0.8 mmol/liter) and low-magnesium seawater (Mg²⁺: 0.1 mmol/liter).

At the end of the experimental period the fish were anesthetized and the blood was collected from the caudal artery into heparinized hematocrit capillaries. After centrifugation of the blood, plasma osmolarity was determined in a Vogel Micro Osmometer. Plasma calcium and chloride were measured by microtitration of 25-μl samples in a Marius calcium titrator and a Marius Chlorocounter, respectively. Sodium and potassium concentrations were determined by flame photometry after 100-fold dilution of 20-μl samples. At the end of the experimental period the osmolarity and sodium, chloride, and calcium concentrations of the experimental media were determined by the same techniques. The protein-bound calcium fraction was determined by ultrafiltration of 100-μl plasma samples (cut off: 1000 M).

For light and electron microscopy the pituitary glands were prefixed in cacodylate-buffered 3% glutaraldehyde (0.1 M; pH 7.4) for 15 min at room temperature. They were fixed in a similarly buffered mixture (1:1:1) of 2% osmium tetroxide, 3% glutaraldehyde, and 5% potassium dichromate, for 1 hr at 0°. They were block-stained for 1 hr in 1% uranyl acetate and embedded in Spurr’s resin. For light microscopy 1-μm-thick sections were stained with toluidine blue and the volumes of cells and nuclei determined as described before (Wendelaar Bonga, 1978a).

For electron microscopy ultrathin sections were poststained with Reynolds’ lead citrate and examined in a Philips EM 300 electron microscope. For quantitative evaluation of the prolactin cells randomly selected samples, totaling about 1000 μm² of cytoplasm per animal, were analyzed. Electron micrographs of these areas with a final magnification of 13,000x were scanned using Kontron Digiplan integration equipment with magnetostriiction tablet. Magnification was calibrated with carbon replica grating. The fractional volumes of the granular endoplasmic reticulum and of the Golgi apparatus were determined by measuring the areas occupied by single strands or stacks of granular endoplasmic reticulum or by Golgi areas. A Golgi area was defined as the cytoplasmic area occupied by a stack of Golgi sacculles and the associated vesicles and presecretory granules. A Golgi field was considered as “active” when electron-dense presecretory material was present within the Golgi sacculles. The fractional volumes of mitochondria and secretory granules (including presecretory granules) were determined, as well as the number of presecretory granules per unit area of cytoplasmic surface.

Differences between the experimental groups concerning the light and electron microscopical data were tested for significance by Wilcoxon’s test. The data on plasma composition were analyzed by Student’s t test. All tests were two sided, at the 5% level.
RESULTS

Prolactin Cells

The prolactin cells are concentrated in the rostral pars distalis. They are interspersed by nongranulated stellate cells. The ultrastructure of both cell types has been described by Dharmamba and Nishioka (1968). Our observations are generally in line with their description.

(a) Freshwater control fish. The rostral pars distalis is large in these fish and constitutes more than one-third of the pituitary gland. The prolactin cells are well developed. They contain only a few mitochondria, but these are relatively large. In an occasional animal the prolactin cells appear to contain only one or two giant mitochondria (Fig. 1). The matrix of such mitochondria often contains electron-dense fibrillar material that may represent microfilaments. The granular endoplasmic reticulum is mostly arranged in large arrays (Fig. 1). The Golgi areas are surrounded by many small clear vesicles, an occasional coated vesicle, and some presecretory granules. In about one-third of the Golgi fields the saccules contain electron-dense fibrillar material that probably represents presecretory substance. These fields are counted as "active." Signs of granule formation by budding from the distal parts of the Golgi membranes are common in active Golgi areas. Many of the presecretory granules in close proximity to the Golgi saccules are considerably smaller (ϕ: 60–100 nm) than the mature secretory granules (ϕ: 140–200 nm). In more peripheral parts of the Golgi fields most of the presecretory granules have attained the same size as the mature granules. They can be distinguished from the latter, since the electron-transparent halo surrounding the dense core is wider than that of the mature granules (Fig. 2). Indications of granule release by exocytosis were occasionally observed (Fig. 1).

(b) Sodium-enriched fresh water. In fish adapted to fresh water enriched with sodium chloride to an osmolarity and sodium concentration as high as that of seawater, the mean size of the prolactin cells has slightly increased, although the difference from the freshwater controls is not significant (Fig. 3). The volumes per cell occupied by nucleus, mitochondria, granular endoplasmic reticulum, or Golgi complex are similar to those of the former group (Figs. 3, 4). The active Golgi areas have increased significantly (P < 0.01) and constitute more than 90% of the total Golgi areas. The number of presecretory granules per unit area of cytoplasm is very high. The number of membrane profiles indicative of granule release is rather low, but higher than in the freshwater control fish.

(c) Calcium-enriched fresh water. Exposure to fresh water containing the same calcium ion concentration as seawater (10.2 mmol/liter) leads to a reduction in the size of the rostral pars distalis, mainly due to a decrease of prolactin cell volume. This reduction is highly significant in comparison with the freshwater controls (P < 0.001; Figs. 3, 5). The nuclei are slightly smaller (P < 0.01). The cells contain several small mitochondria. The volumes per cell occupied by mitochondria (P < 0.001), granular endoplasmic reticulum (P < 0.001), and especially the Golgi system (P < 0.01) are considerably smaller than in the freshwater controls. The volume of active Golgi zones (P < 0.05) and the number of presecretory granules (P < 0.001) are also lower.

Additional light microscope measurements showed that prolactin cell volume in fish adapted for 4 weeks to fresh water enriched with MgCl₂ (5.6 mmol/liter) was slightly, but not significantly, reduced in comparison with freshwater controls (412 ± 62 μm³). Exposure to fresh water containing Mg²⁺ in a concentration similar to that of seawater (56.0 mmol/liter MgCl₂) led to a significant reduction (330 ± 46 μm³; P < 0.01), although the cells were still larger.
Fig. 1. Prolactin cell (freshwater controls) containing extensive granular endoplasmic reticulum (ger) and a giant mitochondrion (mi); ec, release of granules contents by exocytosis.

Fig. 2. Prolactin cell (freshwater controls) showing extensive Golgi fields (Ga) and presecretory granules (arrows); mi, normal mitochondria.
than those of the fish adapted to calcium-enriched fresh water (group c; \( P < 0.05 \)).

(d) Seawater control fish. Prolactin cells and nuclei are slightly smaller than those of group c, but the differences are not significant (Figs. 3, 6). The values for size and activity of the Golgi areas are significantly lower (\( P < 0.001 \)). The differences from the freshwater controls are all highly significant (\( P < 0.001; \) nuclei: \( P < 0.01 \)).

(e) Low-calcium, low-magnesium seawater. If the calcium content of seawater is reduced to that of fresh water, and the magnesium concentration limited to 10% of the seawater value, the prolactin cells are apparently activated. Compared to the seawater controls, cell and nuclear volumes have increased significantly (\( P < 0.001 \) and \( P < 0.05 \), respectively). The volumes per cell of mitochondria (\( P < 0.001 \)), granular endoplasmic reticulum (\( P < 0.05 \)), and total Golgi complex (\( P < 0.01 \)) are twice those of the seawater control fish (Figs. 3, 7). The differences in volume of the active Golgi areas (\( P < 0.01 \)) and the number of presecretory granules (\( P < 0.05 \)) are even greater. The values in general are slightly below those of freshwater control fish.

Additional experiments showed that in fish adapted for 4 weeks to \( \text{Mg}^{2+} \)-free seawater (\( \text{Mg}^{2+} \): 0.1 mmol/liter), prolactin cell volume was similar to that of seawater controls (152 ± 28 \( \mu \text{m}^3 \)). Adaptation for 4 weeks to low-calcium seawater (\( \text{Ca}^{2+} \): 0.8
Fig. 4. Prolactin cell of a fish exposed to sodium-enriched fresh water. The extensive Golgi areas (Ga) contain several presecretory granules (arrows).

Fig. 5. Prolactin cells of a fish exposed to calcium-enriched fresh water. Note the small cell size.
Fig. 6. Prolactin cell of a seawater control fish, showing a small Golgi area (Ga); arrow: presecretory granule.

Fig. 7. Prolactin cell of a fish exposed to low-calcium, low-magnesium seawater, showing extensive granular endoplasmic reticulum and a large Golgi area (Ga) with several presecretory granules (arrows).
mmol/liter) led to an increase of prolactin cell volume (260 ± 61; \( P < 0.01 \)) in seawater fish.

**Plasma Osmolarity and Ion Content**

Exposure to high-sodium fresh water leads to a significant increase in plasma osmolarity \((P < 0.001)\) in comparison with freshwater control values (Table I). Plasma sodium and chloride levels are unchanged, but there is a significant increase in plasma calcium \((P < 0.05)\). In calcium-enriched fresh water, plasma osmolarity has decreased \((P < 0.001)\), whereas sodium, chloride, and calcium remain unchanged. In seawater-adapted fish plasma osmolarity is enhanced \((P < 0.001)\), while sodium and calcium concentrations are similar to those of freshwater fish. Reduction of the calcium and magnesium content of seawater induces a small decrease in plasma osmolarity \((P < 0.01)\) and chloride content \((P < 0.05)\) in comparison with seawater fish. In all groups the percentage of protein-associated calcium amounts to about 45%.

### DISCUSSION

**Prolactin Cells and Environmental Ion Composition**

Prolactin cells of freshwater fish contained significantly more endoplasmic reticulum and mitochondria, while the Golgi apparatus was larger and displayed a significantly higher secretory activity than in seawater fish. These structural differences between the prolactin cells of fresh water and seawater *S. mossambicus* confirm results reported for other euryhaline fish (Schreibman et al., 1973; Batten and Ball, 1977; Wendelaar Bonga, 1978a). Zambrano et al. (1974), studying prolactin cells of *S. mossambicus*, showed that such structural differences reflect considerable differences in secretory activity. During incubation *in vitro*, leucine incorporation as well as prolactin release was considerably higher in prolactin cells from freshwater fish than in those from seawater fish.

The low secretory activity of the prolactin cells of the seawater fish is apparently not related to the high osmolarity or to the

### TABLE I

**Osmolarity and Sodium, Chloride, and Calcium Concentrations of Blood Plasma and Ambient Media**

<table>
<thead>
<tr>
<th></th>
<th>Osmolarity (mOsm/liter)</th>
<th>Na(^+) (meq/liter)</th>
<th>Cl(^-) (meq/liter)</th>
<th>Calcium (meq/liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) FW, controls</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medium</td>
<td>18</td>
<td>6.3</td>
<td>1.5</td>
<td>1.6</td>
</tr>
<tr>
<td>Plasma</td>
<td>345.6 ± 2.3</td>
<td>149.7 ± 4.5</td>
<td>152.8 ± 10.1</td>
<td>6.0 ± 0.8</td>
</tr>
<tr>
<td>(b) FW + NaCl</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medium</td>
<td>1028</td>
<td>502</td>
<td>511</td>
<td>1.6</td>
</tr>
<tr>
<td>Plasma</td>
<td>366.7 ± 2.4</td>
<td>154.6 ± 5.5</td>
<td>161.9 ± 9.3</td>
<td>7.9 ± 1.1</td>
</tr>
<tr>
<td>(c) FW + CaCl(_2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medium</td>
<td>48</td>
<td>4.5</td>
<td>22</td>
<td>20.6</td>
</tr>
<tr>
<td>Plasma</td>
<td>330.3 ± 1.4</td>
<td>148.2 ± 4.2</td>
<td>151.9 ± 7.4</td>
<td>6.0 ± 0.4</td>
</tr>
<tr>
<td>(d) SW, controls</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medium</td>
<td>1044</td>
<td>463</td>
<td>561</td>
<td>20.5</td>
</tr>
<tr>
<td>Plasma</td>
<td>360.6 ± 1.3</td>
<td>147.1 ± 5.6</td>
<td>169.6 ± 13.1</td>
<td>6.2 ± 0.8</td>
</tr>
<tr>
<td>(e) SW, low CA(^{2+})/Mg(^{2+})</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medium</td>
<td>892</td>
<td>459</td>
<td>451</td>
<td>1.7</td>
</tr>
<tr>
<td>Plasma</td>
<td>348.3 ± 6.7</td>
<td>154.3 ± 6.9</td>
<td>146.8 ± 11.8</td>
<td>6.9 ± 0.5</td>
</tr>
</tbody>
</table>

* Values for blood plasma are means ± SD; \( n = 10 \).

* Ambient media, at the end of the experiments: FW, fresh water; SW, seawater.
PROLACTIN CELL ACTIVITY AND CALCIUM

399

The high sodium content of seawater. Prolactin cell activity in high sodium-adapted fish was at least as high as in freshwater fish, although the fish were exposed to a medium similar in osmolarity and sodium content to seawater. There are indications that prolactin secretion in the high-sodium freshwater fish even surpasses that of freshwater fish, as the activity of the Golgi apparatus and the frequency of exocytotic phenomena were the highest found in the present experiments. Reduction of the calcium and magnesium levels in seawater led to an apparent activation of the prolactin cells of seawater-adapted fish, while addition to fresh water of calcium or magnesium ions, in the concentrations typical for seawater, reduced the high prolactin cell activity of freshwater fish almost to the low levels characteristic of prolactin cells in seawater fish. These data show that the increased prolactin secretion following transfer of seawater fish to fresh water is not related to adaptation to a hypoosmotic environment, as suggested by Nagahama et al. (1973), but to adaptation to a medium with a low calcium content. Thus, the calcium concentration is likely the principal environmental factor that controls prolactin secretion in S. mossambicus, just as in the stickleback Gasterosteus aculeatus (Wendelaar Bonga, 1978a, b). In the latter species it was found that not only ambient calcium ions, but also magnesium ions, suppress prolactin secretion, although to a lesser extent. The preliminary observations on the effect of magnesium ions reported here show that these ions also have an inhibiting effect on the prolactin cells of S. mossambicus. However, the reduction of prolactin cell volume induced by 56 mmol Mg^{2+}/liter was less than that of 10 mmol Ca^{2+}/liter.

Prolactin Cells and Plasma Osmolarity

If teleost prolactin cells are incubated in vitro, synthetic activity and prolactin release are stimulated by a reduction of the sodium content of the incubation fluid. This effect has been established for the prolactin cells of various species (Baker and Ingleton, 1975; Benjamin and Baker, 1978), including S. mossambicus (Zambrano et al., 1974; Nagahama et al., 1975; Wigham et al., 1977). It is likely a response to lowered osmolarity of the culture fluid (Nagahama et al., 1975). If seawater fish bearing a pituitary transplant are transferred to fresh water, hormone secretion is enhanced in the grafted prolactin cells (Nagahama et al., 1974; Wigham and Ball, 1977). From such experiments it has been concluded that activation of prolactin cells in vivo following transfer of fish from seawater to fresh water may be due to the fall in plasma osmolarity after transfer. A negative feedback relationship between plasma osmolarity and prolactin cell activity has been proposed (Nagahama et al., 1975). Our results do not reveal a consistent relation between plasma osmolarity and prolactin cell activity. The decrease in plasma osmolarity of seawater fish after transfer to seawater with low calcium and magnesium levels (group e) was indeed accompanied by an increase in prolactin cell activity. However, in freshwater fish exposed to high calcium concentrations a similar decrease in plasma osmolarity was accompanied by a drastic reduction of the secretory activity in the prolactin cells. Moreover, the combined occurrence of high prolactin cell secretion and high plasma osmolarity, in the high sodium-adapted fish, is evidence against the supposition that plasma osmolarity is a factor of major importance in the control of prolactin secretion in S. mossambicus. Our studies on sticklebacks led to the same conclusion (Wendelaar Bonga, 1978a, b). These results show that the reactions to changes in osmolarity of prolactin cells in vitro differ from those of cells in situ.

Prolactin Cells and Plasma Sodium and Calcium

In the present experiments the plasma sodium concentration was rather constant, despite large differences in the external sodium concentrations. This constancy
contrasts with the significant changes occurring in plasma osmolarity, chloride and calcium in some experimental conditions.

In *S. mossambicus*, as in several other teleost species, injection of mammalian prolactin can restore the drop in plasma sodium and osmolarity following removal of the pituitary gland (Dharmamba, 1970). Administration of mammalian prolactin (Craig Clarke, 1972) as well as prolactin isolated from *S. mossambicus* (Farmer et al., 1977) results in a dose-related increase in plasma sodium. In the present long-term experiments however, enhanced prolactin secretion was not correlated with an increase in internal sodium levels, even in the presence of very high external sodium concentrations. On the other hand, plasma total calcium and, probably, plasma ionic calcium proved to be elevated in the high-sodium group. In this group prolactin secretion was very high, and likely surpassing that of freshwater fish. This positive relationship between prolactin secretion and plasma calcium is in line with the supposition that prolactin has specific hypercalcemic capacities in fish. Hypercalcemic effects of prolactin have been reported for killifish (Pang et al., 1973, 1978), eels (Olivereau and Olivereau, 1978), sticklebacks (Wendelaar Bonga et al., 1978), and *S. mossambicus* (Wendelaar Bonga and Van der Meij, 1979). Evidence for a hypercalcemic effect of prolactin in *S. mossambicus* was obtained from studies on specimens with grafted prolactin cells. In intact fish with an additional implanted rostral part of the pituitary gland, plasma sodium concentration was elevated only transiently. However, the concomitant rise in plasma calcium persisted for at least 3 weeks (Wendelaar Bonga and Van der Meij, 1979).

Prolactin Cells and Integumental Permeability

To maintain osmotic and ionic equilibrium in environments as hypertonic as seawater or as hypotonic as fresh water, it is essential that fish keep the permeability of the integument for water and ions at a low level (Evans, 1975). In seawater fish, this is facilitated by the high ambient calcium and magnesium concentrations (Carrier and Evans, 1976). In freshwater conditions, prolactin is likely the factor that reduces water influx and ion outflux over the integument, especially the gill surface (Potts and Fleming, 1971; Carrier and Evans, 1976; Ogawa, 1975, 1977). Earlier reports that prolactin increases water permeability of the skin have been criticized recently (Hirano, 1977). We have suggested before that the activation of the prolactin cells that occur in euryhaline fish after transfer or migration from seawater to fresh water represents a response to the increase of membrane permeability for water and ions caused by the drop in external calcium and magnesium concentrations (Wendelaar Bonga, 1978a, b). Enhanced prolactin release will restore the low permeability of the body surface and in this way facilitate freshwater survival (Evans, 1975). The present results indicate that such a mechanism is not only operating during hyposmotic adaptation but also under hyperosmotic conditions. Seawater fish undergoing reduction of ambient calcium and magnesium concentrations, as well as freshwater fish exposed to high sodium chloride levels, have to cope with enhanced influx of ions and outflux of water. The high prolactin secretion observed under these conditions may lead to a reduction of these fluxes and thus enable the fish to maintain plasma ion levels within normal ranges. This hypothesis is under investigation.

ACKNOWLEDGMENTS

The authors are indebted to Professor A. P. van Overbeeke for critical comments and to Mr. J. H. Visser for technical assistance.

REFERENCES


