Effects of External Mg\(^{2+}\) and Ca\(^{2+}\) on Branchial Osmotic Water Permeability and Prolactin Secretion in the Teleost Fish *Sarotherodon mossambicus*

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

Department of Zoology, University of Nijmegen, 6525 ED Nijmegen, The Netherlands

Accepted November 11, 1982

In *Sarotherodon mossambicus* prolactin cell activity is related to ambient Ca\(^{2+}\) levels, and prolactin has hypercalcemic activity in this species. To study whether prolactin has a direct action on calcium metabolism, or whether prolactin’s relationship with calcium is indirect and connected with control of gill permeability, the effects of external Ca\(^{2+}\) and Mg\(^{2+}\) on prolactin secretion and gill permeability were compared. It appeared that high external Mg\(^{2+}\) was associated with reduced prolactin secretion, even though high Mg\(^{2+}\) resulted in a marked hypocalcemia. Exposure of fish to high Ca\(^{2+}\) levels led to hypercalcemia. Both high Mg\(^{2+}\) and high Ca\(^{2+}\) concentrations in the ambient water reduced the osmotic water permeability of the gills. These results represent further evidence that prolactin secretion in *S. mossambicus* may be affected by any external factor that interferes with branchial permeability. It is concluded that prolactin’s main function in this species is connected with control of branchial permeability rather than calcium metabolism, although internal calcium may be implicated in permeability control.

Prolactin secretion in euryhaline fish is generally much higher in freshwater-adapted fish than in seawater-adapted fish. For the stickleback *Gasterosteus aculeatus* and the cichlid *Sarotherodon mossambicus* (*Tilapia mossambica*) we have related high prolactin secretion in freshwater fish with the low Ca\(^{2+}\) concentration of the water (Wendelaar Bonga, 1978; Wendelaar Bonga and van der Meij, 1980; 1981). Additionally, mammalian prolactin causes slight but distinct hypercalcemia in both species (Wendelaar Bonga *et al.*, 1978; Wendelaar Bonga and Flik, 1982). Similar data have been reported for killifish (Pang *et al.*, 1973; Pang, 1981), eels (Olivereau and Olivereau, 1978), and rainbow trout (Ma and Copp, 1981). Furthermore, as prolonged injection of prolactin increases whole body calcium uptake as well as the degree of mineralization of bones and scales in *S. mossambicus* (Wendelaar Bonga and Flik, 1982), it is suggested that prolactin has a primary influence on calcium metabolism in *S. mossambicus* and, possibly, other teleosts.

Prolactin is, however, considered an important endocrine factor controlling the permeability for water and ions of the integument in freshwater fish and amphibians (Clarke and Bern, 1978; Brown *et al.*, 1981). In *S. mossambicus* prolactin administration reduces the permeability of the integument to sodium (Dharmamba and Maetz, 1972) and the osmotic water permeability of the gills (Wendelaar Bonga and Van der Meij, 1981).

The actions of prolactin on calcium metabolism and on water and ion permeability may, however, be closely related. Integumental permeability is controlled not only by hormones but also by environmental factors, of which the calcium concentration is of primary importance (Potts and Fleming, 1970; Isaia and Masoni, 1976). The effect of external calcium levels on prolactin secretion may be indirect and mediated by the influence of calcium on the permeability of the integument. This is supported by the observation that reduction of ambient calcium concentration leads to an increase in
the osmotic water permeability of the gills 
(Wendelaar Bonga and Van der Meij, 1981). The resulting rise in prolactin secretion may be considered a compensatory response on the part of the fish to achieve appropriate integumental permeability. This interpretation of our data implies that not only the ambient calcium concentration, but any external or internal factor that affects the permeability of the integument for water and monovalent ions will affect the rate of prolactin secretion. Although calcium ions are effective in reducing the permeability for water and ions in a variety of epithelia, magnesium ions may have similar effects (Curran and Gill, 1962; Potts and Fleming, 1970; Isaia and Masoni, 1976). We therefore compared the effects of magnesium and calcium ions on prolactin secretion and osmotic water permeability of the gills in *S. mossambicus*.

**MATERIALS AND METHODS**

Sexually mature male *Sarotherodon mossambicus* averaging 12 cm in length and 20 g in weight were obtained from our laboratory stock. The fish were kept in 100-liter freshwater aquaria at 25° on a 12 hr photoperiod, and were fed throughout the experiments with Tetramin tropical fish food and minced beef heart.

Prolactin cell activity and plasma Mg and Ca levels were determined in fish adapted for 28 days to one of the following solutions:

(a) freshwater (controls); composition in mmol/liter: Na+, 3.0; K+, 0.06; Ca²⁺, 0.8; Mg²⁺, 0.2; Cl⁻, 4.2; SO₄²⁻, 0.5;

(b) Ca²⁺-enriched freshwater; composition as under (a), but with CaCl₂ added to a concentration of 2.5, 5.0, or 10.0 mmol/liter Ca²⁺;

(c) Mg²⁺-enriched freshwater; composition as under (a), but with MgCl₂ added to concentrations of 10.0, 20.0, 35.0, or 54.0 mmol/liter Mg²⁺;

(d) Na⁺-enriched freshwater; composition as under (a) but with NaCl added to concentrations of 54.0 or 10.0 mmol/liter Na⁺.

At the start of the experimental period of 28 days, the salt concentrations of solutions (b)–(d) were increased daily to their final concentration by Day 6 of the experimental period. At Day 28 the fish were slightly anesthetized with MS 222. The blood was collected and the pituitary glands were dissected out.

**Branchial osmotic water uptake rates.** Freshwater fish were adapted for 28 days to high calcium and magnesium levels as described under (b) and (c), but with an osmolality adjusted to 320 mosmol/liter with NaCl. Water inflow rates were determined in isolated gills, after the method described by Ogawa *et al.* (1973). After equilibration for 15 min in physiological saline with an osmolality similar to that of blood plasma (320 mosmol/liter; for composition see Wendelaar Bonga and Van der Meij, 1981), four gill arches per fish were incubated for 30 min in demineralized water. For determination of water flow rates in the absence of an osmotic gradient gill arches were incubated in a solution of NaCl in demineralized water with an osmolality of 320 mosmol/liter. After incubation and freeze-drying the dry weight of the gill arches was determined and the water uptake per milligram dry weight determined.

**Estimation of prolactin cell activity.** For light and electron microscopy the pituitary glands from fish from group (a) and from the highest concentrations of groups (b) and (c) were fixed as described previously (Wendelaar Bonga and Van der Meij, 1980), dehydrated, and embedded in Spurr’s resin. For light microscopy 1-μm-thick sections were stained with toluidine blue and the volumes of the cells determined as described earlier (Wendelaar Bonga, 1978).

To determine the incorporation rate of labeled lysine, the rostral pars distalis (*'prolactin lobe’*: it may contain, however, some ACTH cells) were carefully separated from freshly dissected pituitary glands and incubated for 90 min at 22° in Dulbecco’s Modified Eagle’s Medium (MDM), with 1.25 meq/liter CaCl₂ and 20 mmol/liter Hepes (310 mosmol/liter), and subsequently incubated in 100 μl MDM for 4 hr at 22° in a metabolic shaker (40 Hz) with 10 μCi [³H]-lysine (New England Nuclear Corp., sp act 60 Ci/mol). At the end of the incubation period the lobes were homogenized in 500 μl 0.1 M acetic acid, centrifuged at 10,000g for 5 min, and the supernatant was freeze-dried. The lyophilisate was resuspended in 20 μl buffer for electrophoretic analysis. To the incubation medium 500 μl 20% trichloroacetic acid was added, containing 15 mmol/liter L-lysine. Precipitation occurred overnight at 4° after which the tubes were centrifuged at 10,000g. The supernatant was aspirated and the pellet washed three times with diethyl ether, air-dried, and resuspended in 20 μl buffer for electrophoretic analysis. Sodium dodecyl sulfate (SDS)—polyacrylamide gel electrophoresis was performed using 10 cm-long separating gel consisting of 15% acrylamide, 0.4% methylene bisacrylamide, and 0.1% SDS. The slab gels were stained with Coomassie brilliant blue, dried, and prepared for autoradiography. The autoradiograms were scanned densitometrically. Only traces of ACTH were occasionally found.

**Prolactin administration.** Ovine prolactin (a gift from NIH, Bethesda, Maryland; 30.5 IU/mg) was infused continuously at a constant rate (0.07 μl/hr) for 11 days by intraperitoneally implanted Alzet Osmotic Mini-
pumps model 2001. Five freshwater fish with a body weight of around 95 g received a dose of 0.15 IU/g/day ovine prolactin dissolved in 0.01 N HCl. Five fish receiving solvent releasing minipumps served as controls. The minipumps were implanted under MS 222 anesthesia via an incision in the lateral body wall which was sutured carefully. After 11 days blood was collected and plasma Mg and Ca were determined.

Blood sampling and ion determination. After cutting off the tails of anesthetized fish, blood was collected from the caudal blood vessels in heparinized hematocrit capillaries. After centrifugation of the blood 50-μl samples of the plasma were diluted to 5 ml with distilled water and Mg and Ca concentrations determined, in the presence of lanthanum chloride, using atomic absorption spectrophotometry. Part of the plasma from the fish receiving osmotic minipumps was used for ultrafiltration (membrane cutoff: 10,000 Da). The filtrate was used for determination of the ultrafiltrable Mg and Ca fractions of the plasma.

Statistics. The data were statistically analyzed with Student’s t test (two-sided).

RESULTS

Osmotic water inflow rates of the gills. The long-term effects of Mg and Ca ions on the branchial osmotic water uptake rates were determined in gills of fish adapted to water with the same osmolality as the body fluids. We have shown before that highest osmotic water permeability is found in gills of fish adapted to low-Ca\(^{2+}\) saline with the same osmolality as the body fluids (isosmotic saline; 320 mosmol/liter). Under such conditions the osmotic water flow across the gills is minimal, and this may lead to inactivation of the hormonal mechanisms that control the osmotic water permeability of the integument (Wendelaar and Van der Meij, 1981). Therefore, in this study fish adapted to isosmotic saline were used to test the effects of external Mg\(^{2+}\) and Ca\(^{2+}\) on gill permeability.

Incubation of gills isolated from fish adapted to isosmotic saline (controls) in demineralized water resulted in an almost linear weight increase during the first 30 min. Incubation in isosmotic saline did not result in any weight increase of the gills (Fig. 1a).
A 4-week adaptation of fish to saline with high Mg\(^{2+}\) levels resulted in a significant reduction of the branchial water uptake rate at Mg\(^{2+}\) concentrations above 35 mmol/liter \((P < 0.01; \text{Fig. 1b})\). If compared on an equimolar basis, however, Mg\(^{2+}\) was less effective than Ca\(^{2+}\) in reducing the water uptake rate (Fig. 1b).

**Prolactin cell structure.** Exposure of fish to Mg\(^{2+}\)- or Ca\(^{2+}\)-enriched freshwater for 4 weeks resulted in a reduction of the volume of prolactin cells (Figs 2,3,4a). Although the decrease in cell volume was statistically significant at Mg\(^{2+}\) concentrations of 10 mmol/liter and higher \((P < 0.001)\), equimolar concentrations of Ca\(^{2+}\) had a more pronounced effect (Fig. 4a).

Ultrastructural examination of prolactin cells of fish exposed to 54 mmol/liter Mg\(^{2+}\) showed that, if compared to freshwater controls (0.2 mmol/liter Mg\(^{2+}\)), the extent of the granular endoplasmic reticulum and of the Golgi areas was reduced (Figs. 2,3). While in the controls presecretory granules were commonly found in the Golgi areas, they were scarce in the Mg\(^{2+}\)-treated fish. We found similar changes in prolactin cells from fish exposed to Ca\(^{2+}\)-treated freshwater (10 mmol/liter; Wendelaar Bonga and Van der Meij, 1980; 1981).

To examine whether the reduction observed in prolactin cell volume was caused by the osmolality of the Mg\(^{2+}\) and Ca\(^{2+}\)-containing solutions rather than by a specific effect of these ions, fish were exposed for 4 weeks to saline with osmolalities similar to that of the solution containing 54 mmol/liter Mg\(^{2+}\), the biosynthetic activity was reduced significantly \((P < 0.001)\) to 25 and 21\%, respectively, of the control value (Fig. 4b). In prolactin lobes from fish adapted for the same period to NaCl solution in freshwater with an osmolality similar to that of the solution containing 54 mmol/liter Mg\(^{2+}\), the biosynthetic activity was also reduced significantly \((P < 0.001)\), but only to 60\% of the control value (Fig. 4b).

**\[^{3}\text{H}\]-Lysine incorporation.** Prolactin lobes freshly dissected from freshwater control fish and incubated in a medium containing \[^{3}\text{H}\]-lysine show a marked uptake and incorporation of the labeled amino acid. SDS-polyacrylamide electrophoresis of homogenates of the glands, followed by autoradiography of the gel, revealed two bands with apparent molecular weights of 20,000 and 20,500. Similar bands were found after electrophoresis of the incubation medium, indicating that part of these newly synthesized products were released during incubation. These products likely represent prolactin (Farmer et al., 1977; McKeown et al., 1980).

In prolactin lobes of freshwater control fish, about 6\% of the newly synthesized products were released into the medium during the incubation period (Fig. 4b). In prolactin cells of fish exposed for 4 weeks to 54 mmol/liter Mg\(^{2+}\) or 10 mmol/liter Ca\(^{2+}\), biosynthetic activity was reduced significantly \((P < 0.001)\) to 25 and 21\%, respectively, of the control value (Fig. 4b). In prolactin lobes from fish adapted for the same period to NaCl solution in freshwater with an osmolality similar to that of the solution containing 54 mmol/liter Mg\(^{2+}\), the biosynthetic activity was also reduced significantly \((P < 0.001)\), but only to 60\% of the control value (Fig. 4b).

**Plasma Mg and Ca.** Exposure of fish for 4 weeks to high ambient Mg\(^{2+}\) resulted in an increased total Mg and decreased total Ca level in the blood plasma. Adaptation to high external Ca\(^{2+}\) concentrations led to the reverse, with total plasma Mg levels reduced and total plasma Ca levels enhanced. For fish adapted to the highest Mg\(^{2+}\) and Ca\(^{2+}\) concentrations the differences with freshwater controls were all highly significant \((P < 0.01, \text{Fig. 5a})\).

Administration of ovine prolactin for 11 days to freshwater fish resulted in a significant rise of plasma total Ca \((P < 0.01)\) and a reduction of plasma total Mg \((P < 0.01)\). The nonultrafiltrable plasma Ca and Mg fractions were not notably affected (Fig. 5b).

**DISCUSSION**

**Water Permeability of the Gills**

We determined osmotic water flow across the gills in vitro in demineralized water. This procedure has been frequently used for es-
Fig. 2. Prolactin cell of freshwater-adapted control fish: Ga, Golgi area; ger, granular endoplasmic reticulum (10,400 ×).

Fig. 3. Prolactin cell of fish adapted for 4 weeks to 54 mmol/liter MgCl₂ in freshwater (13,000 ×).
PROLACTIN FUNCTION IN *Sarotherodon mossambicus*

**Fig. 4.** (a) Prolactin cell volume of fish adapted for 4 weeks to varying concentrations of NaCl (Na, – – •; upper scale), MgCl2 (Mg, – – –; lower scale) or CaCl2 (Ca, ■ – ■; lower scale) in freshwater. Means ± SEM of six fish per group. (b) The [3H]-lysine incorporation rate of prolactin lobes incubated *in vitro*. FW: lobes from freshwater adapted fish (controls, 100%); Ca, Mg, Na: lobes from fish adapted for 4 weeks to freshwater containing 10 mmol/liter CaCl2, 54 mmol/liter MgCl2, or 83 mmol/liter NaCl, respectively. Bars represent total incorporation; dense parts: part of total incorporated [3H]-lysine recovered from incubation medium. Means ± SEM of five lobes per group.

**Fig. 5.** (a) Plasma Ca (Ca) and Mg (Mg) levels of fish adapted for 4 weeks to freshwater containing different concentrations of CaCl2 (squares, upper scale) or different concentrations of MgCl2 (dots, lower scale). Means ± SEM of six animals per group. (b) Plasma Mg (left bars) and Ca (right bars) concentrations. Total bars: plasma total Mg or Ca; dense parts: ultrafiltrable Mg or Ca fractions. PRL: fish receiving an osmotic minipump releasing 0.15 IU/g/day of ovine prolactin; ctr, fish with osmotic minipump releasing solvent only. Means ± SEM of 10 fish.
timating osmotic water permeability in fish (Maetz, 1974; Ogawa et al., 1973; Ogawa, 1974, 1977; Isaia and Hirano, 1975; Gallis et al., 1979). Osmotic water flow rates should be determined in the absence of solute-linked water transport. In the present experiments incubation of S. mossambicus gills in saline with the same osmolality as blood plasma did not result in a net flow of water. This indicates that the contribution of solute-linked water transport to the water flow was negligible. The measured net water flow was apparently created by the osmotic gradient across the gills, and therefore reflects the osmotic permeability for water of the gill epithelium. The results show that adaptation of fish to Mg$^{2+}$ levels that are present in seawater (54 mmol/liter) leads to a reduction of the osmotic water permeability of the gills. In this respect Mg$^{2+}$ has the same effects as Ca$^{2+}$. At the same concentrations, Ca ions are more effective than Mg ions, even if it is taken into account that in equimolar chloride solutions the ionic activity of Mg is lower than that of Ca.

As far as the effects of divalent cations on gill permeability are concerned, most attention has so far been paid to Ca$^{2+}$. Reduction of the Ca$^{2+}$ concentration in seawater or freshwater causes an increase in the osmotic or diffusional permeabilities of gills for water and in the permeability for various monovalent ions (Cuthbert and Maetz, 1972; Ogawa, 1974, 1975; Eddy, 1975; Evans, 1975; Pic and Maetz, 1981; Wendelaar Bonga and Van der Meij, 1981). It has also been demonstrated before that in this respect Ca$^{2+}$ can be replaced to some extent by Mg$^{2+}$. For example, in freshwater trout Potts and Fleming (1971) have shown that Mg as well as Ca ions in the water can reduce the integumental permeability for Na$^+$, although Ca ions appeared to be more effective. In seawater-adapted eels transfer to Ca$^{2+}$-free seawater resulted in an increased Na$^+$ turnover rate that was further enhanced if Mg$^{2+}$ was also omitted. Both divalent cations also reduced the osmotic permeability for water and the permeability for chloride of the gills (Isaia and Masoni, 1976).

It is well known that Mg ions, although to a lesser extent than Ca ions, may influence the permeability of cellular membranes in general (Curran and Gill, 1962; Schoffeniels, 1967). Mg and Ca ions bind to phosphate groups of membrane phospholipids, thereby increasing the packing of the lipids and reducing membrane fluidity. As a consequence, membrane permeability is decreased (Ebel and Günther, 1980). Similar processes may account for the effects of Mg and Ca ions on gill permeability.

Control of Prolactin Secretion

Adaptation of fish to freshwater with high Mg$^+$ concentrations leads to a reduction of prolactin secretion that is much more pronounced than that effected by saline solutions of the same osmolality. However, in this respect Ca$^{2+}$ is more effective. The present results are in line with our previous observations on sticklebacks showing that prolonged adaptation to high Mg$^{2+}$ or Ca$^{2+}$ concentrations reduces prolactin cell size (Wendelaar Bonga, 1978). The inhibitory effect of high Mg$^{2+}$ levels on prolactin secretion may explain why adaptation to Ca$^{2+}$-free seawater does not result in a rise of plasma prolactin levels in S. mossambicus, as reported by Nicoll et al. (1981). Seawater contains about 54 mmol/liter Mg$^{2+}$. The same authors also found that plasma prolactin levels do not change following transfer of fish to Mg$^{2+}$-free seawater. This can be explained by the inhibitory effect of the high Ca$^{2+}$ concentration (10 mmol/liter) in seawater. In seawater-adapted S. mossambicus we found activation of prolactin cells only if both Ca$^{2+}$ and Mg$^{2+}$ were reduced simultaneously (Wendelaar Bonga and Van der Meij, 1981). However, we recently failed to demonstrate a similar effect in fish from two strains different from our laboratory stock (unpublished observations). In these fish the prolactin cells re-
sponded to changes in ambient Ca\(^{2+}\) and Mg\(^{2+}\) only in freshwater or saline solutions hypoosmotic to the blood.

The degree of inhibition of prolactin secretion exerted by equimolar concentrations of Ca\(^{2+}\) and Mg\(^{2+}\) parallels the effectiveness of these ions in reducing branchial osmotic water permeability. This result supports our hypothesis that external factors affecting the osmotic water flow across the gills also affect prolactin secretion. This hypothesis was based on observations on the effect of external osmolality on prolactin secretion in *S. mossambicus* (Wendelaar Bonga and Van der Meij, 1981). In fish kept in saline with an osmolality similar to that of the blood, prolactin secretion appeared to be very low. In this situation the osmotic water flow across the gills is also minimal, due to the absence of an osmotic gradient across the gill epithelium. Interestingly, in such fish prolactin cells no longer responded to changes in the external Ca\(^{2+}\) concentration, indicating that the relationship between external Ca\(^{2+}\) and prolactin secretion is indirect.

We suggest that the effects of both Ca\(^{2+}\) and Mg\(^{2+}\) on prolactin cell activity are mediated by their effects on osmotic water flow (and, possibly, on passive ion movements) across the gill epithelium. The main function of prolactin in *S. mossambicus* may be connected with the control of the permeability of the integument to water and possibly, monovalent ions, and not primarily with the control of calcium metabolism. Prolactin is well known for its effects on the permeability of various epithelia in fish (Bern, 1975; Clarke and Bern, 1980) including the lining of the gut, the gills, and the urinary bladder (Hirano *et al.*, 1971; Hirano, 1975; Ogawa, 1975, 1977). In *S. mossambicus* injections of ovine prolactin reduce the passive sodium fluxes (Dharmamba and Maetz, 1972) as well as the osmotic water flow across the gills (Wendelaar Bonga and Van der Meij, 1981). For amphibians evidence is accruing that prolactin has similar effects on the skin and the urinary bladder (Brown *et al.*, 1981).

**Prolactin and Plasma Mg and Ca**

Whereas high Mg\(^{2+}\) levels induced hypocalcemia, and high Ca\(^{2+}\) levels resulted in hypercalcemia, both Mg\(^{2+}\) and Ca\(^{2+}\) inhibit prolactin secretion. This suggests that prolactin is not directly involved in the homeostatic control of plasma Ca. Furthermore, the effects of these ions on prolactin secretion are unlikely to be mediated by changes in plasma Ca levels. In a study on stanniectomized sticklebacks we found a correlation between plasma Ca and prolactin cell activity (Wendelaar Bonga and Greven, 1978). We concluded that prolactin cell activity may be controlled by the plasma Ca level (Wendelaar Bonga and Greven, 1978; Wendelaar Bonga, 1978). The present data make such a control mechanism for prolactin secretion in tilapia very unlikely. Grau *et al.* (1981) came to the same conclusion for plasma calcium after studying prolactin secretion of *S. mossambicus* pituitary glands *in vitro*. However, this certainly does not imply that prolactin does not affect calcium metabolism in this species. Injection of prolactin results in a rise of plasma Ca, as was confirmed in this study. Plasma Mg levels were reduced. Hypercalcemic effects of prolactin have been reported previously for killifish (Pang *et al.*, 1973; Pang, 1981), eels (Olivereau and Olivereau, 1978), trout (Ma and Copp, 1981), and sticklebacks (Wendelaar Bonga *et al.*, 1978). We have furthermore found that in *S. mossambicus* ovine prolactin stimulates whole-body calcium uptake and increases the calcium content of bones and scales (Wendelaar Bonga and Flik, 1982). Thus, although the main function of prolactin may be connected with permeability control rather than calcium metabolism, the hormone nevertheless has pronounced effects on calcium metabolism in this species. We have suggested previously that the effect of prolactin on membrane permeability may be
mediated by internal calcium ions (Wendelaar Bonga and Van der Meij, 1981).

In rainbow trout, European eels (our unpublished results), goldfish (Olivereau et al., 1982), and killifish (Ball et al., 1982), prolactin cell structure appears to be hardly affected by changes in external \( \text{Ca}^{2+} \) concentration. On the other hand, injections of ovine prolactin reduce the osmotic water permeability of the gills of trout, eels, and goldfish (Ogawa et al., 1973; Ogawa, 1974, 1977), and raise plasma calcium in trout (Ma and Copp, 1981), eels (Olivereau and Olivereau, 1978), killifish (Pang et al., 1973; Pang, 1981), similarly as in *S. mossambicus*. This discrepancy can be explained if we assume that there are species-specific differences within the teleosts in the extent to which external calcium affects the permeability of the integument.

ACKNOWLEDGMENTS

The authors are indebted for technical assistance to Mr. A. Coenen and Mr. J. C. Visser, and to Dr. J. C. Fenwick for comments in the course of this study.

REFERENCES


