Effects of Calcitonin on Ultrastructure and Mineral Content of Bone and Scales of the Cichlid Teleost Sarotherodon mossambicus

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Accepted October 28, 1981

Synthetic salmon calcitonin stimulates appositional growth of acellular skeletal bone in S. mossambicus, although it acts rather slowly. Sixteen hours after a single injection of calcitonin in the osteoblasts structural signs of cellular activation became apparent. After 9 days of continuous calcitonin infusion the osteoblasts had increased in size and number and were highly active. Scale formation by the outer scale-forming cells was also stimulated by calcitonin. Conversely, no effects of calcitonin were noticeable on the calcium and phosphate concentrations of the skeletal bone and scale matrix, and no changes were found in total or ultrafilterable plasma calcium levels, or plasma phosphate. The absence of noticeable effects of calcitonin on the concentration of calcium and phosphate in bone and on plasma calcium may be related to the absence of osteocytes and the scarcity or absence of bone resorbing cells in acellular bone. The capacity of calcitonin to induce acute changes in plasma calcium levels by modifying the exchange of calcium between bone and body fluids is therefore limited. If calcitonin performs any function in the plasma calcium regulation of fish with acellular bone, the mechanism of action will be different from that in mammals, where calcitonin is a fast-acting hormone operating predominantly by inhibition of osteocytic osteolysis and osteoclastic bone resorption.

In mammals, a prompt hypocalcemic response is usually observed following injection of calcitonin of mammalian or teleostean origin (Copp et al., 1972; Pang et al., 1971), and because of this calcitonin is considered an important subsidiary hormone in the homeostatic control of blood calcium in these animals (Copp et al., 1972; Cooper et al., 1981). But it is questionable whether calcitonin has a similar significance as a hypocalcemic factor in fish. Indeed, in most studies on fish no effect on plasma calcium was seen after administration of mammalian or fish calcitonin (Pang, 1971; Yamachi et al., 1978; Wendelaar Bonga, 1980; Hirano et al., 1981). Similar findings on higher vertebrates have been rationalized by suggesting that the absence of a hypocalcemic response is due to the presence of such highly efficient homeostatic control mechanisms for calcium that the potential hypocalcemic effect of the hormone is completely masked (Copp et al., 1972). In a study on sticklebacks (Wendelaar Bonga, 1980) we found some support for this explanation, since the prolactin cells, that may have a hypercalcemic function in fish, were activated after prolonged calcitonin treatment. However, an alternative explanation needs consideration. In mammals, the hypocalcemic response to calcitonin is mainly due to the inhibitory effect of the hormone on bone resorption and osteolysis by inhibition of multinuclear osteoclasts and osteocytes, respectively (Mills et al., 1972; Matthews et al., 1972). But as most fish have acellular bone (i.e., the compact bone matrix does not contain enclosed osteocytes) and multinuclear osteoclasts may be absent from acellular bone (Moss, 1962; Weiss and Watabe, 1979), the effect of calcitonin on these cell types in acellular boned fish is obviously precluded. Thus, in fish, calcitonin may be unable to affect the exchange of calcium between bone and blood plasma to an extent comparable to that in mammals.

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In the only ultrastructural study on acellular bone of skeletal origin known to us, osteoblasts and mononuclear bone-resorbing cells were described (Weiss and Watabe, 1979). But whether the degree of mineralization of acellular bone, or the activity of its cells can be influenced by calcitonin is not known. The present study then deals with the effects of synthetic salmon calcitonin not only on plasma calcium and phosphate levels but also on the ultrastructure and degree of mineralization of acellular bone in the cichlid teleost Sarotherodon (Tilapia) mossambicus. Additionally, the scales were studied, as the bone of scales has also been shown to represent a calcium storage pool of physiological importance (Simkiss, 1974; Mugiya and Watabe, 1977). The primary postulate investigated was whether or not the absence of a hypocalcemic response to calcitonin administration in acellular boned fish could be attributed to the absence of any major effects of this hormone on bone resorption, the degree of mineralization of the bone matrix, or both resorption and mineralization.

**MATERIALS AND METHODS**

Sexually mature *S. mossambicus*, with body weights varying between 25 and 49 g, were obtained from laboratory stock. The fish were kept in 100-liter freshwater aquariums at a temperature of 26°, and the fish were exposed to a daily light period of 12 hr. They were fed daily with Tetramin tropical fish food. The fish were exposed to a daily light period of 12 hr. They were under MS-222 anesthesia. The incision was sutured carefully.

After 9 days the fish were killed by mild anesthetization with MS-222 and a blood sample was collected after caudal section from the caudal blood vessels into microhematocrit capillaries. After centrifugation, blood plasma was collected and plasma total calcium and phosphate were determined. An aliquot of blood plasma from each fish was deproteinized by ultrafiltration with a Sartorius Membrane filter to determine the nonprotein bound calcium fraction.

To determine bone mineral content, scales, opercular bones, and tail fins were collected. After cleaning for 2 hr in 1 N KOH, the bony tissue was rinsed three times in 100% ethanol and dried for 16 hr at 90°. After determination of dry weight, the bones were dissolved in 1 ml of 10 N HNO₃ for 2 hr.

Following appropriate dilution of the 10 N HNO₃ plus bone solution, calcium concentrations were measured by microtitration with EGTA in a Marius calcium titrator. Phosphate concentrations were determined with the 1-amino-2-naphthol-4-sulfonic acid procedure in a Technicon autoanalyzer.

For microscopical analysis, central parts of the tail fins and parts of the abdominal body wall were dissected and prefixed for 10 min at 20° in 3% glutaraldehyde in 0.1 M cacodylate buffer at a pH of 7.3. The tissues were then fixed for 1 hr at 0° in a freshly prepared solution of 0.8% OsO₄, 1% glutaraldehyde and 2% K₂Cr₂O₇, in the same buffer. After dehydration in ethanol, the tissues were embedded in Spurr’s resin.

For light microscopy, 1-μm-thick sections were cut with glass knives and stained with toluidine blue. In cross sections of fin rays and scales, cellular and nuclear volumes of osteoblasts and scale forming cells were determined with Kontron Digiplan magnetostriiction tablet. Per animal 50 cells and their nuclei were measured. The number of osteoblasts and scale forming cells per unit length of bone surface was estimated by examining cross sections and determining the number of cells apposing the bone surfaces over a length of 100 mm of bone/animal. Only cell profiles showing nuclei were considered.

For electron microscopical examination, ultrathin sections were cut with diamond knives and stained with Reynolds’ lead citrate. The quantitative data were analyzed for statistical significance with Student’s *t* test at the 5% level.
RESULTS

Calcitonin and Ultrastructure of Bone

Fin rays: Control fish. The structure of fin rays and scales of *S. mossambicus* was described by Lanzing (1976) and Lanzing and Wright (1976). In cross sections of tail fins the fin rays appeared as two apposed semilunar hemisegments covered by a thin periosteal layer which was surrounded by connective tissue. Ultrastructurally, three zones are visible: a central mineralized osseous zone, a slightly mineralized preosseous matrix at the periphery of the osseous zone, and a layer of bone cells. The osseous zone was electron-dense and consisted of compact bone without lacunar spaces, canaliculi, or enclosed osteocytes. The preosseous matrix appeared to be a narrow rim about 1 μm in width and consisted of densely packed collagen fibers and dispersed electron-dense spicular particles which likely represented bone mineral deposits (Figs. 1 and 2). The bone cells in the outer layer were identified as osteoblasts and they formed a single but discontinuous layer at the perimeter of the preosseous matrix (Figs. 1–3). In cross sections of fin rays the bone cells were oval in shape and had a prominent nucleus with condensed chromatin and a narrow rim of cytoplasm. Small cytoplasmic cell processes occasionally penetrated the preosseous matrix. Although some of the osteoblasts appeared to be connected by desmosomes, the majority of cells were not in contact with neighbouring cells. The cytoplasm of the osteoblasts contained some small mitochondria with an occasional small electron-dense granule, some isolated strands of granular endoplasmic reticulum, and small Golgi areas (Figs. 1–3). A characteristic feature of many of these cells was the presence of small electron-transparent vesicles (45–100 μm in diameter) in the vicinity of the outer cell membrane (Fig. 3). Evidence for the presence of bone-resorbing cells was not observed.

Fin rays: Calcitonin treated fish. Seven hours after a single calcitonin injection the fin ray structure of calcitonin treated fish did not differ from that of controls. However, 16 hr after injection, structural differences were observed which suggested an activation of the osteoblasts. In the nuclei, the chromatin was less condensed than in the controls and nucleoli appeared in some nuclei (Fig. 4). Further large electron-dense granules appeared in many of the mitochondria (Fig. 5). In some cells, the cisterns of the granular endoplasmic reticulum were dilated by the presence of electron-transparent material.

After continuous calcitonin treatment for 9 days, marked differences were observed in the periosteal layer. Cellular and nuclear sizes of the osteoblasts had significantly increased (Table 1). The hypertrophy of the cells could be accounted for largely by the increased extent of the granular endoplasmic reticulum and the Golgi areas (Figs. 6–8). Clear vesicles occurred in large numbers at the periphery of the cells (Fig. 8) and signs of extrusion of the contents of these vesicles by exocytosis were commonly observed (Fig. 8). Further, the number of osteoblasts per unit length of bone surface had markedly increased (Table 1) and in the osseous zone, a highly electron-dense rim was visible at the periphery of the hemisegments which was apparently more heavily mineralized than the older bone matrix (Fig. 7). Such rims were never seen in control fish and they are interpreted as evidence of newly formed bone tissue. The thickness of this rim indicated a rate of bone apposition of about 1 μm/day.

Calcitonin treatment for 9 days (0.09 mU g⁻¹ hr⁻¹) did not noticeably influence the calcium or phosphate concentrations of the opercular bone or of the fin rays (Table 2).

Scales: Control fish. The scales consisted of a calcified collagenous matrix with concentrically arranged ridges, the circuli, at the outer surface and a dense layer of unmineralized collagen fibers, the fibrillar
Figs. 1–3. Osteoblasts in the periost of fin rays of mature control fish. The cells are small and contain a few strands of granular endoplasmic reticulum (ger; Fig. 1; $\times 15,700$). Clear vesicles are scarce (arrows; Fig. 1) or absent (Fig. 2, $\times 10,440$; Fig. 3; $\times 11,424$); in the nuclei the chromatin is condensed; poz, preosseous matrix; om, osseus matrix.
Figs. 4–5. Periost of fin rays of fish, 16 hr after calcitonin injection (10 mU/g). The nuclear chromatin is dispersed and some cells have prominent nucleoli (Fig. 4, ×11,000). The mitochondria contain electron dense granules (arrows) and the granular endoplasmic reticulum may be dilated (Fig. 5, ×14,000); poz, preosseous zone; om, osseous matrix.

Fig. 6. Osteoblasts of fin ray periost of fish treated for 9 days with calcitonin. The cells contain cytoplasmic processes (cp) into the preosseous zone (poz). The cytoplasm is extensive and contains well-developed granular endoplasmic reticulum (ger). Clear vesicles are common (arrows); om, osseous matrix (×19,500).
TABLE I

<table>
<thead>
<tr>
<th></th>
<th>Cell area (μm²)</th>
<th>Nuclear area (μm²)</th>
<th>No. of bone-forming cells/mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Osteoblasts*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calcitonin</td>
<td>30.3 ± 4.2**</td>
<td>7.5 ± 0.8*</td>
<td>102.7 ± 8.2**</td>
</tr>
<tr>
<td>Controls†</td>
<td>13.7 ± 2.8</td>
<td>5.5 ± 0.6</td>
<td>64.1 ± 10.7</td>
</tr>
<tr>
<td>Scale-forming cells†</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calcitonin</td>
<td>23.8 ± 0.9**</td>
<td>6.4 ± 0.4*</td>
<td>19.1 ± 4.6</td>
</tr>
<tr>
<td>Controls‡</td>
<td>14.7 ± 1.6</td>
<td>5.1 ± 0.3</td>
<td>16.3 ± 4.2</td>
</tr>
</tbody>
</table>

* Data given as means ± SD of 6 fish/group.
† Osteoblasts in the periost of fin rays.
‡ Synthetic salmon calcitonin administered for 9 days by implanted osmotic minipumps.
§ Hormone-free pumps.
∥ Scale-forming cells at the outer surface of the scales.
* Statistically significant, P < 0.05.
** Statistically significant, P < 0.001.

plates, at the inner surface. In control fish the scale forming cells were found in low numbers and were dispersed over the outer scale surface. The nuclei of these cells were small and often contained condensed chromatin. The scanty cytoplasm contained a few strands of granular endoplasmic reticulum and an occasional mitochondrion (Fig. 9). Golgi areas were rarely observed and electron-transparent vesicles were absent.

Scales: Calcitonin treated fish. The scale forming cells in fish treated for 16 hr or 9 days with calcitonin were almost as low in number as in control fish but the size of the cells and their nuclei had increased significantly (Table 1). The ultrastructure of these cells was similar to that of the calcitonin treated osteoblasts. The cytoplasm contained extensive granular endoplasmic reticulum, and many clear vesicles were present at the cell periphery (Fig. 10). Most active scale forming cells were found at the periphery of the scale. Calcium and phosphate concentrations remained unchanged (Table 2).

Calcitonin and Plasma Calcium Levels

As shown in Table 3, a single injection of 10 mU/g of synthetic salmon calcitonin did not noticeably influence plasma phosphate, plasma total calcium, or the ultrafilterable, nonprotein bound plasma calcium fraction. Plasma Na⁺ and Cl⁻ levels were also unchanged (data not shown here). Higher doses of calcitonin (40 mU/g; data not shown here) were also without effect as was exposure for 9 days to calcitonin administered at a rate of 0.09 mU hr⁻¹ g body wt⁻¹ (Table 3).

DISCUSSION

Calcitonin and Bone Cells

Our observations suggest that synthetic salmon calcitonin stimulates the appositional growth of the acellular bone of *S. mossambicus*. Calcitonin has been reported to exert the same effect on the cellular bone of eels (Lopez et al., 1976) and higher vertebrates (Gaillard, 1970; Mills et al., 1972; Norimatsu et al., 1979). There are, however, marked differences between the mode of action of calcitonin on acellular and cellular bone. As *S. mossambicus* has only compact bone, bone apposition is limited to relatively small areas. Calcitonin appeared to increase the number, size and secretory activity of the osteoblasts in this species but the response of these cells to calcitonin de-
Figs. 7–8. Fin rays of fish treated for 9 days with calcitonin. In the osseous matrix a dense rim of newly synthesized bone is noticeable (nsb, Fig. 7, ×11,200). The osteoblasts contain extensive granular endoplasmic reticulum (ger), and clear vesicles are common (arrows, Fig. 8, ×29,000); poz, preosseous zone; double arrow, exocytosis.

Fig. 9. Scaleforming cell at the outer scale surface of control fish. The cytoplasm contains hardly any cellular organelle; om, osseous matrix of scale (×4750).

Fig. 10. Scaleforming cell at the outer scale surface of fish treated for 9 days with calcitonin; ger, granular endoplasmic reticulum; om, osseous matrix of scale; arrows, clear vesicles (×16,000).
The most conspicuous effect of calcitonin on the cellular bone of higher vertebrates is the immediate inhibition of bone resorption by multinuclear osteoclasts (Mills et al., 1972). In fish, such cells have been observed only in eels (Lopez, 1970). Weiss and Watabe (1979), however, after experimentally induced decalcification, did describe mononuclear cells that they considered to be bone resorbing cells in the acellular bone of *Tilapia macrocephala*. But bone resorbing cells were not observed during the present study on *S. mossambicus*. In mammals, bone resorption is effected not only by osteoclasts, but also by osteocytes and mononuclear macrophages (Heersche, 1978). Intralacunar osteocytes are lacking in *S. mossambicus*, whereas macrophages were absent from the periosteal region. We conclude, therefore, that the action of calcitonin on the bone cells in this species is limited to stimulation of the osteoblasts, at least in the mature, slowly growing fish examined in this study. The presence of bone resorbing cells in rapidly growing juvenile fish cannot be excluded. Like the osteoblasts, the scale-forming cells are activated by calcitonin. These cells are also structurally very similar to osteoblasts.

### TABLE 2
Calcium and Phosphate Contents\(^a\) in Bone from Scales, Operculum and Fin Rays of Freshwater Fish\(^b\)

<table>
<thead>
<tr>
<th></th>
<th>Scales</th>
<th>Operculum</th>
<th>Fin rays</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Calcitonin</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Ca</strong>(^{2+})</td>
<td>4.85 ± 0.45</td>
<td>5.94 ± 0.40</td>
<td>5.76 ± 0.6</td>
</tr>
<tr>
<td>**PO(_4)^{3-})</td>
<td>3.12 ± 0.33</td>
<td>3.61 ± 0.18</td>
<td>3.52 ± 0.29</td>
</tr>
<tr>
<td><strong>Controls</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Ca</strong>(^{2+})</td>
<td>4.97 ± 0.46</td>
<td>5.82 ± 0.43</td>
<td>5.68 ± 0.23</td>
</tr>
<tr>
<td>**PO(_4)^{3-})</td>
<td>3.13 ± 0.14</td>
<td>3.53 ± 0.37</td>
<td>3.51 ± 0.28</td>
</tr>
</tbody>
</table>

\(^a\) Millimoles per gram dry weight.

\(^b\) Fish were treated for 9 days with synthetic salmon calcitonin (*calcitonin*) or solvent (*controls*), administered by implanted osmotic minipumps. Means ± SD of 6 fish/group. Differences from the controls are statistically nonsignificant.

### TABLE 3
Effect of Synthetic Salmon Calcitonin on Plasma Total Calcium, Plasma Ultrafilterable (non-protein-bound) Calcium, and Plasma Phosphate (mmol/liter) of Freshwater *S. mossambicus*\(^a\)

<table>
<thead>
<tr>
<th></th>
<th>Total calcium</th>
<th>Ultrafilterable calcium</th>
<th>Phosphate</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Calcitonin, 7 hr</strong> (^b)</td>
<td>2.96 ± 0.20</td>
<td>1.48 ± 0.21</td>
<td>0.64 ± 0.05</td>
</tr>
<tr>
<td><strong>Controls</strong></td>
<td>2.92 ± 0.09</td>
<td>1.58 ± 0.13</td>
<td>0.57 ± 0.04</td>
</tr>
<tr>
<td><strong>Calcitonin, 16 hr</strong> (^c)</td>
<td>3.01 ± 0.27</td>
<td>1.61 ± 0.17</td>
<td>0.62 ± 0.06</td>
</tr>
<tr>
<td><strong>Controls</strong></td>
<td>3.09 ± 0.31</td>
<td>1.68 ± 0.27</td>
<td>0.54 ± 0.03</td>
</tr>
<tr>
<td><strong>Calcitonin, 9 days</strong> (^d)</td>
<td>2.90 ± 0.32</td>
<td>1.49 ± 0.22</td>
<td>0.65 ± 0.05</td>
</tr>
<tr>
<td><strong>Controls</strong></td>
<td>3.08 ± 0.13</td>
<td>1.53 ± 0.14</td>
<td>0.59 ± 0.04</td>
</tr>
</tbody>
</table>

\(^a\) Data are given as means ± SD of 10 fish/group. The differences between calcitonin treated fish and controls are statistically nonsignificant.

\(^b\) Single injection 7 hr before blood sampling; dose 10 mU/g.

\(^c\) Single injection 16 hr before blood sampling; dose 10 mU/g.

\(^d\) Osmotic minipump implanted 9 days before blood sampling; dose 0.09 mU g\(^{-1}\) hr\(^{-1}\).
Calcitonin and Bone Mineralization

Prolonged calcitonin treatment did not noticeably influence the total concentration of calcium and phosphate of the bone, although the thin layer of newly formed bone may be more heavily mineralized than the preexisting bone matrix. An increased calcium and phosphate content was observed, however, in cellular bone from seawater eels after prolonged calcitonin treatment (Lopez et al., 1976) and similar effects of calcitonin on bone mineralization were reported for birds and mammals (Baud et al., 1970; Bélanger and Copp, 1972). In mammals, the enhanced mineralization following calcitonin treatment is ascribed to the inhibitory effect of calcitonin on the osteolytic activity of the osteocytes. As a consequence, the continuous deposition of calcium and phosphate at free crystalline bone surfaces, a noncellular event, is no longer compensated by osteocytic osteolysis, thus leading to enhanced bone mineral content (Mills et al., 1972). Acellular bone has been considered relatively inaccessible to the physicochemical type of calcium and phosphate exchange with the body fluids (Norris et al., 1963; Simmons et al., 1970). This is supported by observations on the marine toadfish (Opsanus tau) where a very low rate of calcium exchange was observed between the acellular skeletal bone and the body fluids (Simmons et al., 1970). Unfortunately, such data is not available for freshwater fish that live in low calcium environments. But there is some evidence that the degree of bone mineralization in freshwater fish can be reversibly altered by hormones. For example, in S. mossambicus prolonged prolactin treatment leads to a significant increase in calcium and phosphate in skeletal bones as well as scales (Wendelaar Bonga and Flik, 1982). Moreover, the scales of fish with cellular as well as acellular bone are known to represent an important store of calcium and phosphate that can be mobilized under conditions of starvation or sexual maturation (Simkiss, 1974). Demineralization of scales can be induced by administration of estrogens (Mugiya and Watabe, 1977). Apparently, the exchange of calcium and phosphate between acellular bony tissues and body fluids is controlled primarily by hormones other than calcitonin.

Calcitonin and Plasma Calcium

We were unable to demonstrate a hypocalcemic response to calcitonin administration in freshwater and seawater acclimated S. mossambicus. Neither acute nor prolonged effects of the hormone were observed. In freshwater sticklebacks we did report a transient but significant decrease in the nonprotein bound plasma calcium fraction 7 hr after a single injection of synthetic salmon calcitonin (Wendelaar Bonga, 1981). Further, Mathur (1979) was able to induce hypocalcemia in the catfish Channa punctatus, 1 hr after injection of porcine calcitonin. Acute (Chan et al., 1968) as well as prolonged (Lopez et al., 1976) hypocalcemic responses to mamalian or synthetic salmon calcitonin also have been reported in European eels. In many other studies on eels and various other fish species no effects on plasma calcium could be demonstrated (Pang, 1971; Copp et al., 1972; Yamauchi et al., 1978; Wendelaar Bonga, 1980; Hirano et al., 1981). The absence of a noticeable hypocalcemic response, however, as in the present experiments on S. mossambicus, does not imply that the hormone has no significance as a factor in the homeostatic control of plasma calcium. Hypocalcemic effects following calcitonin administration are absent in birds and are often barely noticeable in adult mammals with mature skeletal tissue. Yet in these animals a role of calcitonin in plasma calcium homeostasis seems well established (Cooper et al., 1981) and the failure to find frank hypercalcemia following calcitonin treatment is probably due to the fact that the hypocalcemic action of calcitonin is compensated directly by the
strong hypercalcemic action of parathyroid hormone (Copp et al., 1972). Only in young mammals, with high bone cell activity, do hypocalcemic effects become clearly evident. In these animals the reduction of cell mediated calcium release following calcitonin administration leads to a rapid but transient drop in plasma calcium as calcium deposition in the bone is stimulated by the hormone (Matthews et al., 1972).

In fish, potential experimentally induced hypocalcemia may be attenuated by the enhanced release of a hypercalcemic hormone, such as prolactin (Pang et al., 1978; Wendelaar Bonga et al., 1981). In this respect it is of interest that prolactin cell activity was enhanced in sticklebacks after prolonged treatment with calcitonin and that plasma calcium levels were unaltered (Wendelaar Bonga, 1980). But even if calcitonin is involved in plasma calcium homeostasis in fish, its mechanism of action in fish with acellular bone is clearly different from that in mammals, since our results make it likely that calcitonin has no capacity to inhibit calcium release from bony tissues, whereas the enhanced rate of bone formation by the osteoblasts following calcitonin injection seems to occur too slowly to have acute effects on plasma calcium levels.

ACKNOWLEDGMENTS

The authors are indebted to Dr. J. C. Fenwick for critical comments, to Mr. J. C. A. van der Meij and Mr. J. H. Visser for technical assistance, and to Mrs. E. M. Jansen-Hoorweg for assistance in the preparation of the manuscript.

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