Results and discussion. Freeze-cleaving splits plasma membranes producing 2 fracture faces[14,15]: face PF (or A) directed toward the exterior of the cell, and face EF (or B) directed toward the cytoplasm[16]. Fracture faces PF and EF of embryonic myocardial cell plasma membranes are studded with many 6–10 nm particles (as measured in replicas) distributed at random (Figure 1).

Thus far the investigation has shown the earliest signs of nexus formation to be at 10 dpc. At that time the faces of PF of the plasma membranes frequently show linear arrays of particles (consisting of 6 to 20 particles) and sometimes aggregates made up of 2 or 3 rows of linear arrays (Figure 1). Much less frequently, one can observe small clusters of particles in hexagonal arrays with a 9–10 nm center-to-center spacing with the corresponding fracture face EF showing corresponding arrays of 35–50 nm depressions (Figure 2). These structures are characteristic of nexuses. Sometimes, nexuses faces PF (averaging 50 to 90 nm in width), show, in the central areas, one or two approximately circular zones containing only a few widely spaced particles (Figure 2). At 12 dpc both linear and hexagonal arrays of particles are found on the faces PF and both are more common than at 10 dpc. The smallest nexuses, usually oval or circular in form, sometimes have 'arms' formed by one or two rows of particles (Figure 3). At 14 dpc, on the fracture faces PF, linear arrays or particles have become rarer, whereas the hexagonal arrays are now relatively numerous. Their size (on average 250 nm in the greater length) is also larger than at 10 and 12 dpc. The nexuses faces EF appear studded with small pits, hexagonally arrayed (Figure 4).

The establishment of nexuses at this very early stage suggests that this type of intimate intercellular contact with low electrical resistance may be an important feature in the development of the heart as a whole, particularly in the establishment of good electrical contact for the passage of the depolarizing impulse.

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Localization of Wheat Germ Agglutinin Receptor Sites on Yeast Cells by Scanning Electron Microscopy

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Summary. WGA receptor sites on the surface of *Saccharomyces cerevisiae* cells pretreated with an α- mannanase were localized by gold granules labelled with WGA. The receptor sites were found on the bud scars, the mother cell-bud junction (chitin) and the bud, but not on the mother cell.

Recently we have developed a new technique for the visualization of cell surface receptor sites by scanning electron microscopy (SEM) using gold granules labelled with antibodies[1] We report here the localization of wheat germ agglutinin (WGA) receptor sites on the cells of *Saccharomyces cerevisiae* by SEM. WGA is a lectin (MW 26 000) whose combining site is complementary to sequences of β-(1→4)-N-acetyl-D-glucosaminyl units[2,3] which in budding yeasts are predominantly found in the chitin of the bud scars[4–7]. However, a significant amount of glucosamine is also found elsewhere in the cell wall[5,6,8] as in the mannan protein. The main constituents of *S. cerevisiae* cell walls are a β-glucan (49%) and the α-mannan-protein (40%)[5] whose polysaccharide moiety is in the outer layer of the wall[9]. The cell wall mannan is attached through N-acetyl-β-D-glucosamine (possibly in the form of di-N-acetylchitobiose) to asparagine units of the protein[10].

WGA receptor sites appeared on the surface of yeast cells only when the cells were first treated with an α-mannanase known to cleave most of the α-(1→2)- and α-(1→3)-linked side chains of *S. cerevisiae* mannan[11].

Experimental. Labelling of gold granules. Gold granules could not be labelled and stabilized against flocculation with WGA[1] due to its too low molecular weight. WGA was therefore cross-linked to bovine serum albumine (BSA). WGA (L'Industrie Biologique Française, 1 mg) and BSA (4 mg) were dissolved in 0.005 *M* NaCl (0.25 ml) and the solution was neutralized to pH 7 with 0.2 *N* K_2CO_3. The proteins were cross-linked with 0.25% glutaraldehyde (0.05 ml)[12]. After 2 h at 25 °C, 0.005 *M* NaCl (12.2 ml) was added.

Colloidal gold granules (Sol. I) of a size suitable for SEM[1] (50 nm) were prepared according to Frens (Sol C of ref.[13]). The optimum amount of protein necessary to label 5 ml of Sol I was determined by adding diluted WGA-BSA solutions (1 ml). After 1 h at 25 °C, too small an amount of protein caused agglutination of the colloid, seen as a decrease of the absorbance at 540 nm.

Gold granules (Sol II) of a size suitable for TEM (5 nm) were prepared according to FAULK and TAYLOR[14]. The optimum amount of protein necessary to label 5 ml

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of Sol II was determined by adding diluted WGA-BSA solutions (0.5 ml) until the colloid could no longer be flocculated by 10% NaCl (0.5 ml).

Sol I and II (100 ml) were labelled with a 10% excess of WGA-BSA and Concanavalin A using procedures described previously[1,14]. The markers were finally suspended in *Tris*-buffered saline, pH 7.0 (10 ml) containing 0.001 *M* Mg^{+2} and Ca^{+2} Mn^{+2} and 0.5 mg Carbowax 20-M (Union Carbide Chemicals Co.) filtered with a Millipore filter (0.45 µm pore size) (Buffer A). The WGA gold colloids were shown to have retained their activity as chitin (Fluka) and human erythrocytes were readily marked. Due to their size they mark only cell surface receptor sites.

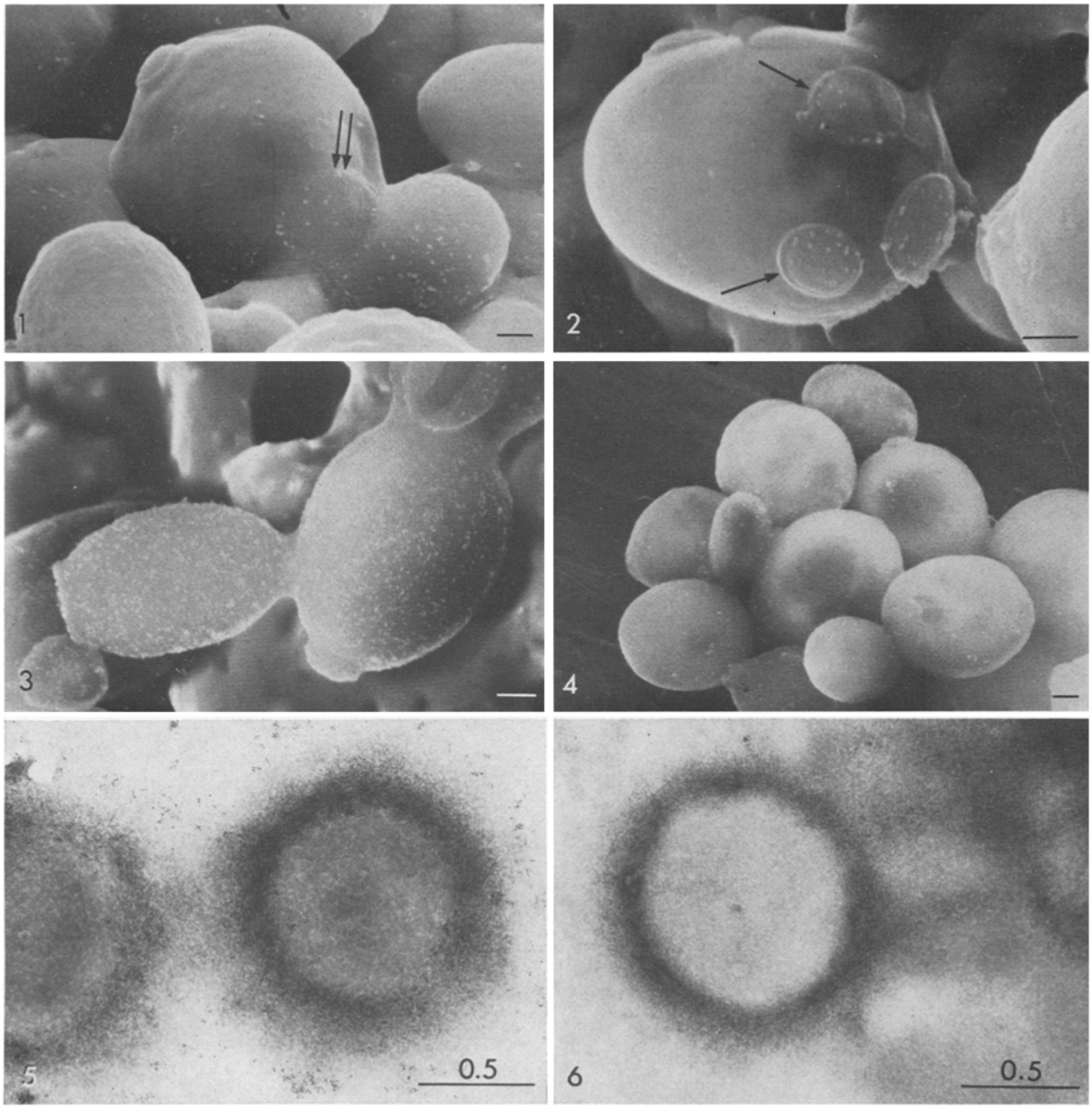

Fig. 1 and 2. α-Mannanase treated *S. cerevisiae* cells marked with WGA. The cells in buffer A ($A_{650\ nm}$ 0.55, 0.9 ml) were incubated for 3 h with WGA (Sol I, $A_{530\ nm}$ 8.6, 0.1 ml). The cells were centrifuged at 2000 rpm (1 min) and washed with buffer A (2×). The bud scars (single arrow), the mother cell-bud junction (double arrow) and the bud wall are marked. Figure 1: ×5,200, Figure 2: ×8,500.

Figure 3. *S. cerevisiae* cells marked with Con A (Sol I) using similar conditions to those of Figures 1 and 2. The marker was homogeneously distributed on all cells. ×6,000.

Figure 4. A group of α-mannanase treated *S. cerevisiae* cells incubated with Con A (Sol I). As expected, label was much reduced. ×3,750.

Figure 5. *S. cerevisiae* bud scars marked with WGA. Bud scars ($A_{650\ nm}$ 0.4, 0.05 ml) were incubated for 3 h with WGA (Sol II) ($A_{520\ nm}$ 3.6. 0.2 ml) in buffer A (0.75 ml). The bud scars were centrifuged at 3000 rpm (2 min) and washed with buffer A (2×). The WGA receptor sites (chitin) appear on the ring structure. × 36,000.

Figure 6. A control experiment. Bud scars were incubated as in the Legend of Figure 5 in the presence of penta-acetyl chitopentaose (2 mg/ml). No label was observed. × 36,000.

The cells were examined after critical point drying, in a Cambridge S 4–10 Stereoscan as described previously[1]. Bud scars were examined after negative staining with a Phillips EM 300 electron microscope as described by Bauer et al.[5].

Preparation of yeast cells and bud scars. *S. cerevisiae* was grown and prefixed with glutaraldehyde as described previously[1]. The cells were suspended in either Buffer A or in 0.05 *M* phosphate buffer, pH 6.8 (2 ml, $A_{650\,nm}$ 5.0) that contained 0.2 µmole $CaCl_2$, 0.1 mg BSA and *Arthrobacter* α-mannanase[11] (0.0027 unit[15]). The suspensions were incubated for 20 h at 25 °C. The amount of mannose released in the supernatant corresponded to 12% of the cell's total sugars[16].

Bud scars were prepared from *S. cerevisiae* cell walls by the action of a β-(1→3)glucanase as described previously[5] and suspended in water.

Results and discussion. Intact *S. cerevisiae* cells could not be marked with WGA (Sol I) indicating that WGA receptor sites were not exposed on the cell surface. However, when the cells were treated with the α-mannanase, WGA receptor sites were located on the bud and bud scars but not on the mother cell (Figures 1 and 2). The receptor sites could have been attributed either to chitin, chitin oligomers, the N-acetyl-D-glucosamine link of the mannan-protein or glycolipids containing di-N-acetyl chitobiose which have been found in membrane preparations[17]. The various possibilities were tested against the following informations: Chitin has been shown to be present only in the bud scars[5]. In control experiments, WGA marking was not only totally inhibited by penta-acetyl chitopentaose[18] (2 mg/ml) but also by the mannan-protein (2 mg/ml) prepared enzymatically by action of a β(1→3) glucanase on the cell walls[19] indicating that in solution the WGA marker reacted with the mannan-protein. As WGA is specific for diacetyl chitobiose or higher chitin oligomers[20], this confirmed that the mannan-protein link is a diacetyl chitobiose (or a higher oligomer)[10] and that the protein moiety must lie deep in the cell wall[9] since intact cells were not marked. However, it is doubtful that the WGA receptor sites found on the bud are located in the mannan-protein, since both bud and mother cells were homogeneously marked with ConA (Sol I) which reacts with the side chains of mannan (Figure 3). When the side chains were removed with the α-mannanase, marking was diminished (Figure 4). Glycolipids containing diacetyl chitobiose[17] could not be localized by WGA on *S. cerevisiae* protoplasts, although membrane mannan was well marked with ConA[21]. Therefore the nature of the WGA receptor sites on the bud (Figure 1) is still unknown.

Bud scars prepared enzymatically contain chitin and mannan[5]. They showed WGA receptor sites mainly on the ring structure when examined by transmission electron microscopy (Figure 5). No label was observed when penta-acetyl chitopentaose (2 mg/ml) was present in the labelling mixture (Figure 6). Under the same conditions, marking of mannan with ConA (Sol II) was weaker. Therefore the WGA receptor sites on the bud scars must be attributed to chitin. This is further supported by the fact that chitin synthesis begins at the onset of budding[22] which is shown by the WGA marking at the mother cell-bud junction (Figure 1, double arrows)*.

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New Karyological Data of *Rhinoderma*: the Chromosomes of *Rhinoderma rufum*[1]

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Summary. The chromosomes of the Chilean frog *Rhinoderma rufum* are described for the first time. This chromosome set is compared with the karyotype of *R. darwinii*. The importance of the karyological data applied to the phylogeny and systematics of this genus are discussed. A tentative hypothesis of karyological evolution of *Rhinoderma* is given.

Amongst frogs of the superfamily Bufonoidea[4], familiar status and phylogenetic relationships of *Rhinoderma* are controversial[4–6]. The frogs of this genus, endemic of the cool and humid forest of Southern Chile, have a unique life history among the Anuran-tadpoles development in the male vocal sacs. In recent years, karyological data have been an important tool for phylogenetic and systematics studies. From this point of view, some authors[7,8] studied the chromosomes of *Rhinoderma darwinii* and concluded that the genus belongs to the family Leptodactylidae and shows karyological affinities with the Telmatobinae species[7].

Until recently, only one species of *Rhinoderma* was known (*R. darwinii*); however, FORMAS et al.[9] added another species (*R. rufum*) to the genus demostrating the true identity of the enigmatic Chilean frog *Heminectes rufus* Philippi 1902. The 2 species are different in morphology of the feet and developmental patterns.

In this paper, the chromosomes of *R. rufum* are described for the first time. This chromosomal set is compared with the karyotype of *R. darwinii*, which is here redescribed. The importance of the karyological data applied to the phylogeny and systematics of *Rhinoderma* are discussed. A tentative hypothesis of karyological evolution of *Rhinoderma* is given.

The frogs used in this study included: 8 males and 12 females of *R. darwinii* from the vicinity of Valdivia

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