fava bean is present in certain local diets leading to very high fitness of the G-6-PD homozygote. Careful surveillance of local diets for such a possibility would be of interest.

Summary. Although glucose-6-phosphate dehydrogenase deficient individuals may suffer (sometimes fatally) from favism, a high incidence of this trait occurs in many Mediterranean populations. This apparent paradox is explained on the basis of a synergistic interaction between favism and G-6-PD deficiency that provides increased protection against malaria compared to that of the G-6-PD deficiency alone. This relationship is analogous to that between various hemoglobins and malaria in that there is selection for a more severe trait if it provides more protection against malaria.

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Colloidal Gold Granules as Markers for Cell Surface Receptors in the Scanning Electron Microscope

Several techniques have been developed recently in order to visualize cell surface receptor sites by high resolution scanning electron microscopy (SEM). The peroxidase-diaminobenzidine reaction product (easily detected by TEM) is quite difficult to distinguish from surface differentations[1], whereas immunolatex spheres[2,3] and haemocyanin molecules[4] appear to be better suited as markers for SEM observations. However, the preparation of these immunospecific labels is laborious. We report here a simple method colloidal gold granules as visible markers, a technique which has already given satisfactory results in TEM[5–8].

Experimental. Homogeneous populations of colloidal gold granules of different particle sizes can be easily prepared[9]. The granules carry a net negative charge and can be coated by addition of proteins such as Concanavalin A or antibodies, the binding being probably of non-covalent nature. However, protein-coated gold granules

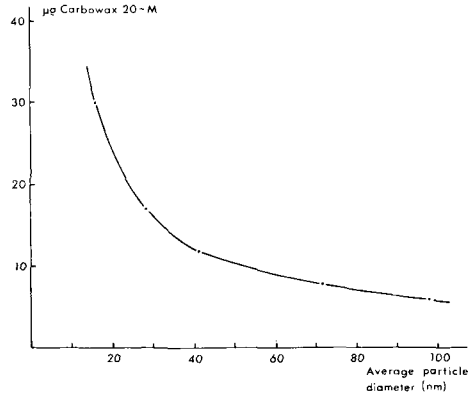

Fig. 1. Stabilization of colloidal gold by polyethylene glycol. A series of monodisperse gold sols of increasing particle diameter (16–97.5 nm as determined by TEM) was prepared[9] and neutralized to pH 7.0 by addition of 0.2 *M* K_2CO_3 (pH-paper, not pH-meter as the electrode is readily contaminated with the colloid). Colloidal gold (5 ml) was mixed with 1 ml water containing increasing amount of Carbowax 20-M (Union Carbide Chemicals Co.). After 1 min, a 10% NaCl solution (1 ml) was added. The absorbance was measured after 5 min at 525 to 540 nm (maximum of the uncoated colloid). A S-shaped curve was constructed from which the minimum amount of Carbowax necessary to fully stabilize the colloid against flocculation was determined.

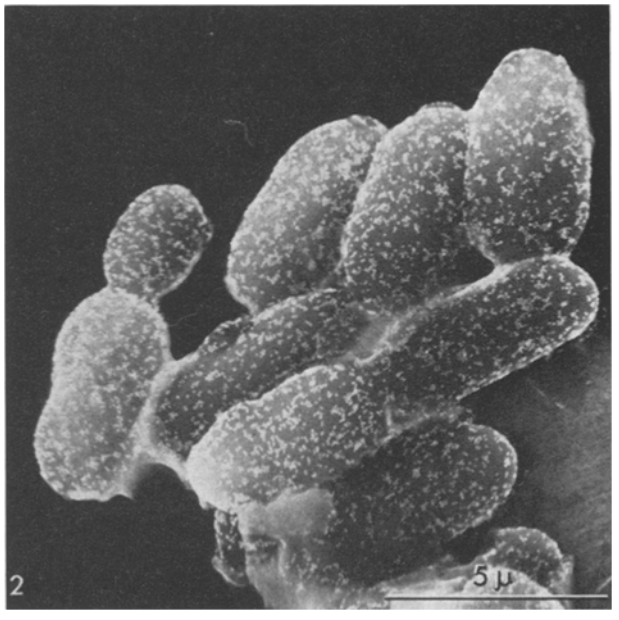

Fig. 2. A group of *C. utilis* cells marked with the total anti-*C. utilis* antiserum adsorbed to 60 nm colloidal gold particles. The antiserum was dialyzed overnight at 5 °C against 5 m*M* NaCl (pH 7.0). The optimal amount of protein to stabilize the colloid against flocculation by NaCl was determined as described in the legend of Figure 1, using the antiserum instead of Carbowax 20-M. The antiserum (1.8 mg) was added, with stirring, to colloidal gold (200 ml, sol B of ref.[9]). After 1 min, a 1% Carbowax solution in water (2 ml) was added and the colloid neutralized to pH 7.0 with 0.2 *M* K_2CO_3. The colloid was centrifuged at 10'000 rpm for 30 min at 5 °C. The supernatant was sucked off and the pellet redispersed in 20 ml *Tris*-buffered saline (pH 7.5), containing 0.2 mg/ml Carbowax 20-M. The yeast cell suspension was incubated in the same buffer at 25 °C for 1 h with an excess of the colloid. The suspension was centrifuged at low speed and washed with the buffer. The yeast cells were rapidly dehydrated in increasing concentrations of ethanol (2–3 min) and finely dispersed on a conductive copper adhesive, mounted for SEM aluminium stubs. The preparations were examined without metal coating in a Cambridge S4-10 Stereoscan, at an accelerating voltage of 30 kV at a tilt angle of 45°.

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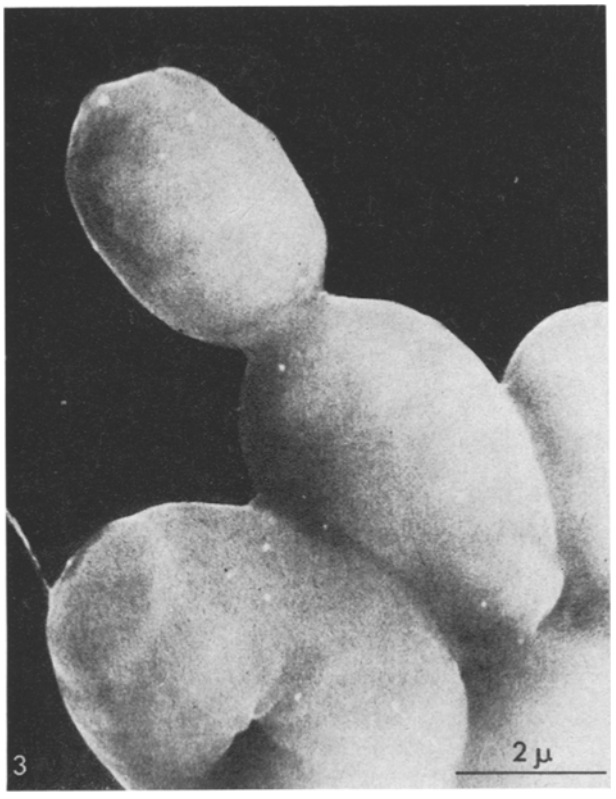

Fig. 3. *S. cerevisiae* cells incubated with the total anti-*C. utilis* antiserum. Pratically no label is observed.

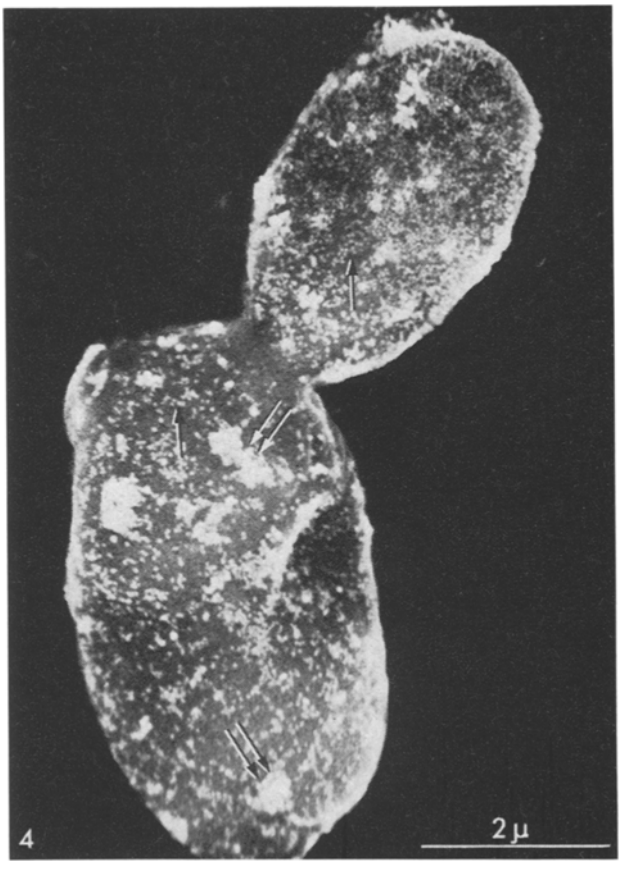

Fig. 4. Double labelling experiment of *C. utilis* cells. First marker: Concanavalin A adsorbed to 40 nm gold particles (arrows); the colloid was in *Tris*-buffered saline (pH 7.0) containing 2 µmole $MnCl_2$/ml and 0.5 mg/ml Carbowax 20-M. Pratically no label was observed when the cells were incubated in the buffer containing also 5 mg/ml methyl α-D-mannopyranoside. Second marker: Anti-nonmannan antibodies adsorbed to 80 nm gold particles (double arrows). The colloids were prepared as described in the legend of Figure 2.

of suitable diameter for SEM flocculated slowly in presence of an excess of protein or in various buffers. We found that the flocculation of either uncoated or coated gold granules could be completely prevented when a small amount of polyethylene glycol was present (Figure 1). Appropriate control experiments showed that nonspecific adsorption could thus be completely eliminated by using either a specific inhibitor or colloidal gold stabilized with polyethylene glycol.

Candida utilis cells grown to the logarithmic growth phase in a yeast extract-glucose medium (1.5% glucose, 0.5% yeast extract) were harvested by centrifugation and fixed in *Tris*-buffered saline (pH 7.0) containing 3% glutaraldehyde. After several washings in the above buffer, they were marked with colloidal gold particles (40, 60 and 80 nm in diameter as determined by SEM) coated with either a total anti-*C. utilis* antiserum, purified anti-mannan antibodies, anti-nonmannan antibodies[7] or Concanavalin A as described in the legend of Figure 2. Control experiments were carried out with the total anti-*C. utilis* antiserum directed agains *S. cerevisiae* cells (grown and prepared identically to *C. utilis*) or with methyl α-D-mannopyranoside, a potent inhibitor of Concanavalin A.

Results. A group of *C. utilis* cells marked with the total anti-*C. utilis* antiserum adsorbed to 60 nm gold particles is shown in Figure 2. Note that the marking is random but quite homogeneous over the whole cell wall surface. About 70% of the receptor sites can be attributed to the cell wall mannan. This is deduced from labelling experiments with Concanavalin A and purified anti-mannan antibodies. It also corresponds with TEM observations previously reported[7]. The bud cell shows a slightly denser label than the mother cell which is attributed to an active synthesis and incorporation of mannan into the newly forming bud cell wall[6,7]. Practically no marking can be detected when the total anti-*C. utilis* antiserum is directed against *S. cerevisiae* cells (Figure 3). As colloidal gold particles of different sizes can be prepared, a cytochemical double marking can be achieved. In Figure 4, *C. utilis* cells were first marked with Concanavalin A (specifically indicating mannan) absorbed to 40 nm gold granules. Anti-nonmannan antibodies bound to 80 nm particles were then applied as a second marker. The Concanavalin A showed again a random but homogeneous distribution of the mannan, whereas the anti-nonmannan components appeared to be arranged in patches.

Discussion. Colloidal gold particles are therefore quite suitable for direct SEM visualization of cell surface receptor sites by means of adsorbed phytohaemagglutinins or immunoproteins. We have shown that colloidal gold particles can be labelled with enzymes such as catalase and with polysaccharides such as yeast mannan. Erythrocytes and their ghosts have also been successfully labelled with colloidal gold particles coated with Concanavalin A. These results will be published elsewhere and indicate that this labelling technique appears to be general. The advantage of a heavy metal label, compared to organic markers is mainly its strong emission of secondary electrons, and therefore it can easily be distinguished from cell surface differentiations (lower emission of secondary

electrons) when uncoated preparations are examined. In addition the metallic marker permits good spatial resolution to be achieved in SEM[10].

Summary. A rapid method has been developed to visualize cell surface receptors in the SEM. Thus mannan at the surface of *Candida utilis* cells was localized by stabilized colloidal gold granules coated with either anti-mannan antibodies or Con A.

[10] Acknowledgment. We thank Mrs M. WEBER for the photographic work.

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Morphology of Colloidal Gold, Ferritin and Anti-Ferritin Antibody Complexes

Electron microscopical studies on the formation of ferritin – anti-ferritin complexes have been published previously[1–3]. Colloidal gold – ferritin mixtures have also been used to demonstrate membrane holes in osmotic and saponin hemolyses and membrane lesions in immune lysis[4, 5]. However, the structure of the complexes formed between colloidal gold particles, ferritin and anti-ferritin antibodies has not been studied in detail. It was therefore interesting to investigate the morphology of these complexes in order to evaluate their potential use as markers for transmission – and scanning-electron microscope cytochemical studies.

Experimental. The anti-ferritin antibodies were isolated by immunoadsorption from a colostral lactoserum of a cow immunized with horse spleen ferritin (cadmium free, 2× crystallized; Pentex Biochemicals). The presence of precipitating anti-ferritin antibodies was shown by a positive ring test[6] against a ferritin solution (1 mg/ml).

Ferritin and anti-ferritin antibodies were adsorbed onto colloidal gold according to the procedure of FAULK and TAYLOR[7], modified by GERBER et al.[8]. The colloids were suspended in phosphate-buffered saline, pH 7.2 to a final absorbance of 3.6 at 520 nm.

The different preparations were examined in a Philips EM 300 electron microscope after negative staining with 5% aqueous uranyl-acetate.

Results. In Figures 1–3 the individual materials used in our experiments are represented. The non-stabilized colloidal gold particles tend to aggregate (Figure 1). They have a polyhedrical form (inset) and are unstable under the electron beam. With progressive beam radiation, they become electron transparent (mass loss). The horse spleen ferritin (Figure 2) is quite homogeneous; however, some detached apoferritin (protein shell of the ferritin molecule) can be observed occasionally (arrow). The purified anti-ferritin antibodies (Figure 3) form small clusters of fairly constant diameter (30–40 nm). Aggregates of variable sizes of ferritin marked with colloidal gold granules (average diameter 5.2 nm) are visualized in Figure 4. Over 95% of the ferritin molecules are labelled with at least one gold particle. Adsorption of the gold colloid takes place onto the protein part of the ferritin molecules. Free apoferritin is therefore labelled as well. Figure 5 illustrates the purified anti-ferritin antibodies marked with colloidal gold. Most important is the observation that all colloid particles are covered by antibodies, i.e. after negative staining a clear, electron-transparent zone is visible around all colloidal gold granules. In favourable projections, the Y-shape of the anti-ferritin antibodies adsorbed onto the colloid particles can be revealed (Figure 5 simple arrow and inset). It is evident that this arrangement with the colloid granules in the center, surrounded by adsorbed immunprotein, is most favourable for immunocytochemical reactions. Small clusters of free antibodies (compare with Figure 3) are recognized occasionally (double arrows). Complexes between ferritin and colloidal gold, coated with anti-ferritin antibodies, are seen in Figure 6. They appear, due to the presence of antibodies, less densely packed than those formed by ferritin and uncoated colloidal gold. In this preparation, rather important fluctuations in the size of the colloid granules occurred. Finally, the rather heterogeneous complexes formed between ferritin marked with colloidal gold and colloidal gold covered with anti-ferritin antibodies are demonstrated in Figures 7 and 8. With adequate concentrations of ferritin and anti-ferritin antibodies, complexes of the type seen in Figure 7 are obtained. All ferritin molecules are surrounded by colloid particles, but it is impossible to distinguish between the gold granules directly adsorbed to the ferritin and those linked to the ferritin via the anti-ferritin antibodies. In Figure 7, the ferritin and anti-ferritin antibodies are not revealed because no negative staining was applied. Nevertheless, the ferritin nucleus (simple arrow) is easily distinguished from the gold particles (double arrow) by its considerably lower density. The same complexes after negative staining with uranyl acetate are shown in Figure 8. Again all the ferritin molecules are surrounded by colloid granules. The typical ferritin structure is masked (arrow) due to adsorption of antibodies. This is in agreement with observations on ferritin – anti-ferritin complexes[1].

Discussion. From the electron micrographs presented it is evident that mixed complexes are very heterogeneous in size and therefore not suitable as cytochemical markers. Two observations are, however, of importance. Firstly protein molecules are adsorbed around colloidal gold granules in a sterically favourable position necessary for cytochemical coupling or for the antibody-antigen reaction. This property has already been successfully exploited in labelling experiments in the transmission-[7–10] and

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