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LECTIN RECEPTOR SITES ON RAT LIVER CELL NUCLEAR MEMBRANES

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SUMMARY

The presence and localization of lectin receptor sites on rat liver cell nuclear and other endomembranes was studied by light and electron microscopy using fluorescein and ferritin-coupled lectin conjugates.

Isolated nuclei labelled with fluorescein-conjugated Concanavalin A (Con A) or wheat germ agglutinin (WGA) often showed membrane staining, which sometimes was especially bright on small stretches of the nuclear surface. Unlabelled nuclei and nuclei with a complete ring fluorescence were also seen. The nuclear fluorescence corresponded in intensity to that seen on the surface of isolated rat liver cells.

Con A-ferritin particles were seldom detected on the cytoplasmic surface of the intact nuclear envelope. However, at places where the 2 leaflets of the envelope were widely separated or where the outer nuclear membrane was partly torn away, heavy labelling was seen on the cisternal surface of both the inner and outer nuclear membranes. Labelling with Con A-ferritin was also found on the cisternal side of rough endoplasmic reticulum present in the specimens. No labelling was seen on the cytoplasmic surface of mitochondrial outer membrane.

The results demonstrate the presence of binding sites for Con A and WGA in nuclei and an asymmetric localization of these sites on the cisternal side of ribosome-carrying endomembranes in rat liver cells.

INTRODUCTION

The external surface of mammalian cells is rich in glycoproteins and glycolipids (Cook & Stoddart, 1973; Hughes, 1973). Biochemical and ultrastructural methods have been widely used for the characterization of these surface components. Lectins capable of binding to cell surface oligosaccharides have proved to be especially valuable as specific surface probes of cellular membranes (Nicolson, 1974*a*; Sharon & Lis, 1974). Concanavalin A (Con A) and wheat germ agglutinin (WGA) have been extensively applied to the study of cell surface structure of normal and malignant cells (Burger, 1973; Nicolson, 1974*b*). These lectins have different binding specificities. Con A binds to α-D-glucopyranosyl, α-D-mannopyranosyl and related saccharide groups (Goldstein, 1975) and WGA to *N*-acetyl-glucosamine containing oligosaccharides (Allen, Neuberger & Sharon, 1973; Nagata & Burger, 1974).

Less is known about the lectin-binding sites on endomembranes of mammalian cells. In addition, different methods have yielded conflicting results regarding the presence and distribution of these sites within the cells. Agglutination of isolated bovine liver

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cell nuclei and mitochondria by 5 lectins, including Con A and WGA, has been taken as evidence for the presence of Con A-binding sites on the nuclear surface. Further evidence is offered by binding of radioactively labelled Con A to isolated rat liver cell nuclei (Kaneko, Satoh & Ukita, 1972). On the other hand, Con A-peroxidase has been claimed to label not only the nuclear surface but also the cisternal surfaces of both the outer and the inner nuclear membranes (Monneron & Segretain, 1974).

Ultrastructural studies with ferritin-conjugated lectins have led to a third view on the localization of lectin receptor sites on intracellular membranes. Only an asymmetric labelling of the cisternal surface of rough endoplasmic reticulum vesicles was obtained in homogenates of mouse myeloma cells (Hirano *et al.* 1972). Similarly, Con A-ferritin particles have been found to bind only to disrupted membranes from chromaffin granules, not to intact granules (Eagles, Johnson & van Horn, 1975).

In order to shed more light on the localization of intracellular lectin-receptor sites we studied the binding of fluorescein-coupled Con A (FITC-Con A) and WGA (FITC-WGA) and of ferritin-conjugated Con A (Con A-ferritin) to isolated rat liver cells, their isolated nuclei and other endomembranes.

MATERIALS AND METHODS

Isolation of rat liver cells and nuclei

Rat liver cells were isolated from normal adult Sprague-Dawley rats using a method described by Seglen (1973). The rat livers were first perfused with a calcium-free buffer and then with a buffer containing 0·5 % collagenase (Worthington). Nuclei were isolated by homogenization of the excised and minced livers in 0·32 M sucrose-3 mM $MgCl_2$ solution and purified by centrifugation through a sucrose step gradient as described earlier (Virtanen & Wartiovaara, 1974). In the final fraction this method yields small amounts of other endomembranes also, especially rough endoplasmic reticulum vesicles and mitochondria.

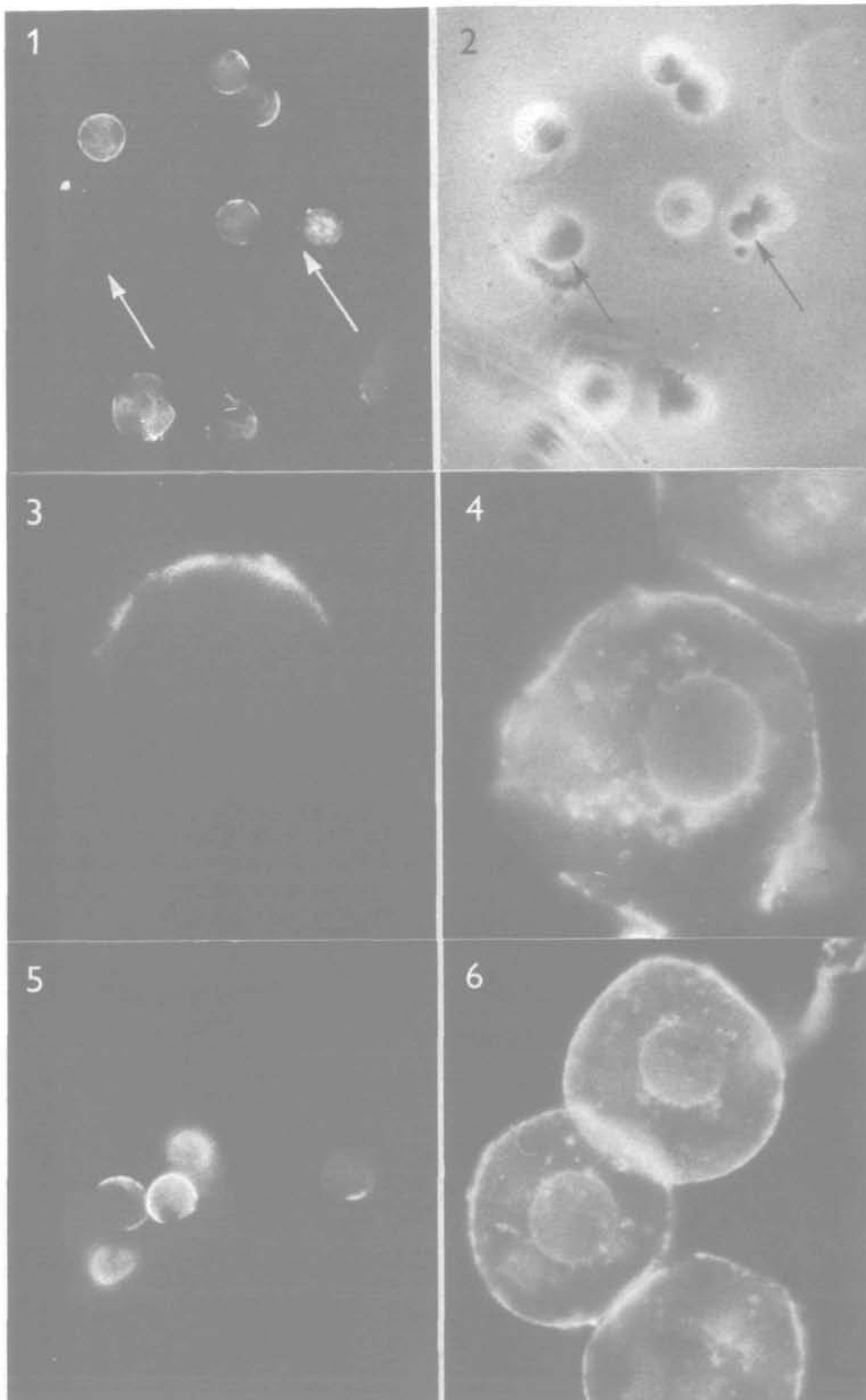
Figs. 1, 2. Isolated rat liver nuclei fixed in paraformaldehyde, stained with fluorescein-coupled Concanavalin A (FITC-Con A) and photographed under fluorescence (Fig. 1) and phase-contrast (Fig. 2) microscopy. Most of the nuclei show partial surface fluorescence, and the one in the upper left-hand corner shows complete ring fluorescence; two (arrows) totally lack fluorescence. × 1500.

Fig. 3. Higher magnification of nucleus with part of the surface showing fluorescence of varying intensity after FITC-Con A labelling. × 7000.

Fig. 4. Isolated rat liver cells labelled with FITC-Con A after paraformaldehyde fixation. In addition to fluorescence of the surface membrane, granular mid-cytoplasmic and clear nuclear surface fluorescence can be seen. × 4000.

Fig. 5. Isolated rat liver cell nuclei fixed in paraformaldehyde and labelled with fluorescein-coupled wheat germ agglutinin (FITC-WGA). Varying fluorescence is seen on the nuclear membranes. × 1500.

Fig. 6. Isolated rat liver cells labelled with FITC-WGA after paraformaldehyde fixation. The bright surface fluorescence resembles that obtained with FITC-Con A. Granular cytoplasmic and nuclear surface fluorescence is also seen. × 2400.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Labelling studies with Con A and WGA conjugates

For fluorescence microscopy the specimens were fixed for 30 min at 0 °C in 4 % paraform-aldehyde-PBS (phosphate-buffered saline, pH 7·2), washed 3 times in PBS at room temperature and incubated for 30 min at room temperature with fluorescein isothiocyanate-Con A (FITC-Con A, MILES, Lot No. 70/24) or fluorescein isothiocyanate-WGA (FITC-WGA, MILES Lot No. FWG-9) suspended in PBS at a concentration of 100 μg/ml. After incubation the specimens were washed 3 times in 20 vol. of PBS and mounted for fluorescence microscopy in 30 % glycerol buffered with PBS (pH 8·5). Fluorescence and phase-contrast photographs were taken with a Zeiss Universal microscope equipped with a Zeiss III RS epi-illuminator (barrier filters KG1, KP500, KP490, dicroid mirror FT510 and omission filter LP250). Kodak Tri-X (Kodak, Rochester) or Agfapan 400 (Agfa, Leverkusen) films were used for photography.

For ultrastructural studies the isolated cell and nuclear preparations were fixed for 30 min at 0 °C in 2·5 % glutaraldehyde buffered with 0·1 M sodium cacodylate (pH 7·2), washed thoroughly 4 times in cold PBS and incubated for 30 min at 0 °C with a commercial Con A-ferritin conjugate in PBS (1·2 or 2·4 mg of protein/ml; Calbiochem, prepared by the method of Nicolson & Singer, 1971). Incubated nuclei were washed 3 times with 0·2 M sodium cacodylate buffer (pH 7·2), postfixed for 60 min in 1·5 % osmium tetroxide in 0·1 M phosphate buffer (pH 7·2), and embedded in Epon 812. Thin sections were either lightly stained with lead citrate or left unstained for easier visualization of the labelling pattern. Electron micrographs were taken with a JEOL 100B electron microscope at 60 or 80 kV.

The specificity of Con A labelling in fluorescence and electron microscopy was tested by pre-incubating the conjugates with 0·2 M α-methyl-D-mannoside (Calbiochem) for 30 min at room temperature. The specificity of WGA labelling was tested by preincubation of the conjugate in 0·2 M *N*-acetyl-glucosamine (Sigma) in PBS for 30 min at room temperature before labelling. Preincubation with increasing concentrations of unlabelled Con A (Pharmacia Fine Chemicals) or WGA (Sigma) resulted in a decrease and finally in a lack of labelling with fluorescent lectins at a 10-fold excess of unlabelled lectin (1 mg/ml).

RESULTS

Immunofluorescence studies with FITC-Con A and FITC-WGA

Isolated nuclei labelled with FITC-Con A often showed bright fluorescence on short stretches of the nuclear surface (Figs. 1, 3). However, also nuclei with ring-like fluorescence were seen. Some nuclei visible by phase-contrast microscopy were completely negative with respect to FITC-Con A fluorescence (Figs. 1, 2). Isolated cells similarly labelled with FITC-Con A showed a continuous surface membrane

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Fig. 7. Isolated rat liver cell nucleus fixed with glutaraldehyde and labelled with Con A-ferritin. Only very few Con A-ferritin particles are seen on the intact nuclear membrane. *inm*, inner nuclear membrane; *onm*, outer nuclear membrane; *r*, ribosomes. × 75 000.

Fig. 8. Nucleus with the 2 leaflets of the nuclear envelope slightly separated from each other. Con A-ferritin particles are seen on the cisternal surfaces of the nuclear envelope. *inm*, inner nuclear membrane; *onm*, outer nuclear membrane. × 70 000.

Fig. 9. Dense labelling with Con A-ferritin of cisternal surfaces of both inner (*inm*) and outer (*onm*) nuclear membranes is seen at places where both membranes have been widely separated. Only very few ferritin particles are seen on the surface of the outer nuclear membrane. × 28 000.

Fig. 10. Labelling with Con A-ferritin is seen at a place where outer nuclear membrane (*onm*) has been torn away. The surface of the outer nuclear membrane adjacent to this area has no Con A-ferritin label. *inm*, inner nuclear membrane. × 81 000.

fluoresence and often a granular midcytoplasmic and a distinct nuclear surface labelling (Fig. 4).

Rat liver nuclear preparations incubated with FITC-WGA were labelled mostly on short stretches of the nuclear surface (Fig. 5) although ring-like staining and non-fluorescent nuclei were also encountered as in specimens treated with FITC-Con A. FITC-WGA labelled isolated liver cells showed ring-like surface fluorescence in addition to cytoplasmic and nuclear surface staining (Fig. 6).

Ultrastructural studies with Con A-ferritin

In the nuclear preparations labelled with Con A-ferritin most of the nuclei were ultrastructurally well preserved with intact membranes and identifiable nuclear pores. Cytoplasmic organelles, especially rough endoplasmic reticulum and mitochondria, were occasionally found among the nuclei and were also studied for Con A-ferritin binding.

Nuclei with seemingly intact membranes bound only a few Con A-ferritin particles on the surface of their outer membrane (Fig. 7). Similar haphazard labelling was seen in the nuclear pore regions and in the nuclear interior.

In nuclei where the 2 leaflets of the nuclear envelope were separated Con A-ferritin labelling was seen on cisternal surfaces of both the inner and the outer nuclear membrane (Figs. 8, 9). Dense labelling with Con A-ferritin particles was also seen on the inner nuclear membrane at places where the outer nuclear membrane was torn away (Fig. 10). In partially tangential sections of isolated nuclei, clusters of Con A-ferritin could be seen only outside ribosome-covered areas representing the surface of the outer nuclear membrane (Fig. 11).

Rough endoplasmic reticulum membranes identifiable by their attached ribosomes had Con A-ferritin particles on their cisternal surfaces, but not on the ribosome-carrying surfaces (Fig. 12). No labelling was seen on the surface of mitochondria although membrane vesicles adjacent to the mitochondria could be densely covered with Con A-ferritin (Fig. 13). No conclusions can be drawn from the labelling of these vesicles due to their unidentifiable nature.

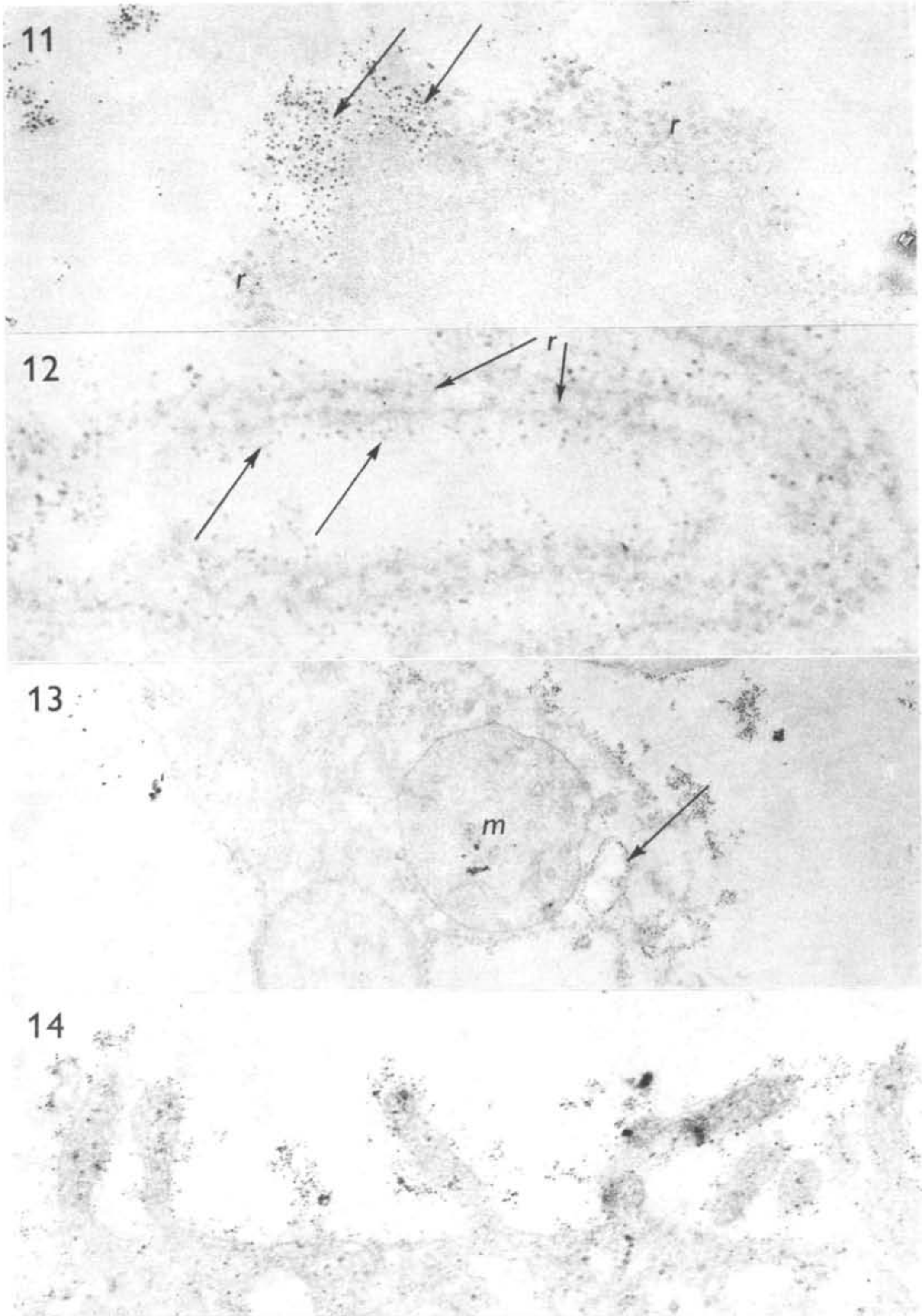
Isolated rat liver cells were found to have a microvillous surface heavily studded with Con A-ferritin comparable in amount to that seen on the labelled endomembranes described above (Fig. 14).

Fig. 11. Tangential section of isolated nucleus labelled with Con A-ferritin. Ferritin particles (arrows) are seen only outside areas covered with ribosomes (*r*) which represent the surface of the nuclear envelope. × 68000.

Fig. 12. Endoplasmic reticulum membranes in the nuclear fractions have Con A-ferritin label (arrows) on the opposite sides to ribosomes (*r*). × 80000.

Fig. 13. Outer membrane of mitochondrion (*m*) lacks Con A-ferritin particles although nearby membrane vesicles show dense labelling (arrow). × 32000.

Fig. 14. Isolated rat liver cell stained with Con A-ferritin has abundant label on the surface. × 58000.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Inhibition of lectin labelling

In inhibition experiments both with 0·2 M α-methyl-D-mannopyranoside in the case of FITC-Con A and Con A-ferritin labelling, and with 0·2 M *N*-acetyl-glucosamine in the case of FITC-WGA, no labelling of either whole cells or endomembranes was obtained indicating high specificity in the observed binding of lectins.

DISCUSSION

Rat liver cell nuclei and nuclear membranes have been extensively characterized by biochemical and cytochemical means (cf. Franke, 1974; cf. Kasper, 1974). However, many aspects of their composition are still to be clarified, mainly due to difficulties caused by contaminating cell organelles and plasma membrane fragments (cf. Kasper, 1974). As an example, contradictory volumes for nuclear content of glycoproteins have been reported (Kashnig & Kasper, 1969; Kawasaki & Yamashina, 1972; Phillips, 1973).

A further approach has been the use of lectins as specific probes for membrane oligosaccharides. Such lectin receptors have been demonstrated also in nuclear preparations in various ways including lectin-induced nuclear agglutination (Nicolson, Lacorbière & Delmonte, 1971) and binding of radioactively labelled lectins to nuclei (Kaneko *et al.* 1972). However, due to the easily disruptable double membrane structure of the nuclear envelope, conclusions drawn on the localization of the lectin receptors on nuclear membranes have had to be treated with caution owing to lack of ultrastructural evidence on distribution of the binding sites.

In the present study paraformaldehyde and glutaraldehyde fixation were used to prevent nuclear disruption and lectin receptor movement on nuclear membranes during incubation. These fixatives have been reported to have no effect on the binding of Con A or WGA to cell surface membranes (Inbar *et al.* 1973; Noonan & Burger, 1973). The controls for the specificity of the lectin binding are of course also crucial. Inhibition of lectin binding with α-methyl-D-mannopyranoside in the Con A experiments and with *N*-acetyl-glucosamine in the WGA experiments indicated the specificity of the observed fluorescence and ferritin labelling.

Our experiments with fluorescein-conjugated Con A and WGA resulted in a continuous labelling of isolated rat liver cell surface. In isolated nuclei a disrupted staining was often obtained, which can be explained by the ultrastructural results with Con A-ferritin labelling: there seems to be a simple correlation between the presence of intact nuclear membranes and lack of fluorescence and ferritin binding. The Con A-ferritin labelling observed in the nuclei differs from the results obtained with Con A-peroxidase staining of calf thymus nuclei (Monneron & Segretain, 1974). In the latter the label was localized in addition to the cisternal surfaces of the nuclear envelope also on the surface of the outer nuclear membrane. This discrepancy could well be explained by the unspecific binding of peroxidase to cellular membranes as suggested by Nakane (1975).

Rough endoplasmic reticulum (RER) found in the specimens also bound Con A-ferritin on the cisternal surfaces, which is in agreement with the studies by Hirano

et al. (1972). Using Con A-ferritin and ricin-ferritin labelling they found only asymmetric cisternal surface binding to RER vesicles. Similarly Con A-ferritin particles have been shown to bind asymmetrically to membranes derived from isolated chromaffin granules of bovine adrenal glands but not to intact granules. This suggests that only the internal surfaces of these granule-limiting membranes are able to bind lectins (Eagles *et al.* 1975). Asymmetric distribution of lectin receptors on cytoplasmic membranes has also been suggested in a study on the binding of ^{3}H-Con A to membrane proteins derived from rat liver cell subcellular fractions including RER and nuclear membranes (Keenan, Franke & Kartenbeck, 1974). The surface of the membrane surrounding the azurofil and specific granules of rabbit polymorphonuclear leucocytes could be of a different nature, as the membrane has been claimed to bind ricin-ferritin particles (Feigenson, Schnebli & Baggiolini, 1975).

Thus the ribosome-carrying membranes, the nuclear surface and the RER seem to have on their cisternal surface and not on their ribosome-carrying surface binding groups at least for Con A and WGA indicating the presence of α-D-glycopyranosyl and/or α-D-mannopyranosyl groups and *N*-acetyl-glucosamine. As liver cell nuclei have been claimed to lack glycolipids (Kleinig, 1970) the lectin-binding sites in the membranes are probably part of glycoproteins. We have previously demonstrated lack of staining of the surface of isolated rat liver cell nuclei with colloidal iron hydroxide, suggesting the absence of sialic acids (Virtanen & Wartiovaara, 1974). Whether the cisternal of the ribosome-carrying endomembranes of these cells have in addition to the first saccharides also terminal sugars remains still an open question.

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