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Recycling of the asialoglycoprotein receptor: biochemical and immunocytochemical evidence

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[Plate 1]

One of the best documented systems of receptor-mediated endocytosis is the clearance of asialoglycoproteins (ASGP) from the blood plasma by liver parenchymal cells. There are 200000–500000 ligand binding sites per cell, which makes this system favourable for molecular studies of receptor function. By using both biochemical and immunocytochemical approaches, we have obtained evidence for receptor recycling. We have also localized the intracellular site at which the endocytosed receptor and ligand dissociate.

The human hepatoma cell Hep G2 contains abundant ASGP receptors (approximately 225000 per cell). In growing cells approximately 85% of the functional receptors are on the cell surface and the remaining 15% are internal. The maximal rate of ligand uptake in this cell system at 37 °C is approximately 30000 molecules per cell per minute. Each functional receptor can therefore bind and internalize more than 50 ligand molecules during a 6 h period (in the absence of new receptor synthesis), or one ligand each 8 min.

To follow both ligand and receptor during their common endocytosis and to visualize the compartment in which the dissociation of ligand from receptor occurs, we have used our recently developed double-labelling immunocytochemical electron microscopic techniques with purified antibodies against ASGP ligand and ASGP receptor. In normal rat hepatocytes, both ligand and receptor are taken up from the sinusoidal cell surface in clathrin-coated vesicles. Both receptor and ligand are associated with the membrane of small clathrin-coated vesicles close to the cell surface. Larger vesicles, farther removed from the surface, contain ligand accumulated within the lumen. The membranes of these larger vesicles contain little receptor, but receptor was concentrated in detached vesiculotubular extensions, which were largely free of ligand. These vesicles represent the compartment of uncoupling of receptor and ligand (Curl) during their common endocytosis. Ligand contained within the vesicle lumen is then transferred to multivesicular bodies and lysosomes; the tubular extensions may carry receptor back to the cell surface.

Introduction

Many cells are capable of internalizing molecules by receptor-mediated endocytosis (r.m.e.). R.m.e. involves binding of a ligand, such as a hormone, virus, plasma protein, or toxins, to specific receptor molecules functionally exposed at the cell surface. Receptor–ligand complexes generally accumulate in coated pits of the plasma membrane followed by internalization of the complexes in small membranous vesicles (Goldstein *et al.* 1979). Many ligands such as α_2-macroglobulin, low-density lipoprotein and insulin, are transported within membrane-limited compartments to lysosomes, where they are rapidly degraded (Pastan & Willingham

1981). Within acidic endocytic vesicles, the membrane of enveloped viruses fuses with the vesicle membrane, enabling the virus core to reach the cytoplasm (Helenius *et al.* 1980). In many cases, the receptors are spared lysosomal degradation and return to the cell surface.

We and others have been studying the receptor for galactose-terminal carbohydrates of glycoproteins (asialoglycoprotein (ASGP)), which is localized in the hepatic parenchymal cell (Ashwell & Harford 1982). Endocytosis of asialoglycoproteins has been studied in considerable

TABLE 1. SPECIFICITY OF [^{125}I]ASOR† BINDING TO HEPATOMA CELLS

(Dishes were washed and incubated with [^{125}I]ASOR (2 μg ml^{-1}) in the absence or presence of additional agents in the standard manner (see Schwartz *et al.* 1981 *a*). Non-radioactive asialoorosomucoid, orosomucoid and asialogalactoorosomucoid were added at 200 μg ml^{-1}. EDTA was present at 5 mM. Neuraminidase preincubation was performed by incubation with 20 milliunits in 1 ml of phosphate-buffered saline (p.b.s.) for 15 min at room temperature. Results are expressed as the percentage of the total [^{125}I]ASOR bound compared with the control ('none').)

addition	[^{125}I]ASOR bound (%)
none	100
EDTA	13
none/EDTA*	29
asialoorosomucoid	22
orosomucoid	93
asialogalactoorosomucoid	84
neuraminidase preincubation	24

* One set of dishes was allowed to bind [^{125}I]ASOR in the standard manner and was thereafter rinsed and incubated for 5 min at 4 °C with p.b.s. containing 5 mM EDTA.

detail in whole liver *in vivo*, in perfused liver *in situ*, and in isolated rat hepatocytes (Ashwell & Morell 1974; Hubbard *et al.* 1979; Steer & Ashwell 1980; Baenziger & Fiete 1980; Schwartz *et al.* 1980). There are as many as 500000 high-affinity surface receptors per hepatocyte (Schwartz *et al.* 1980; Zeitlin & Hubbard 1982). In addition, this receptor has been isolated and purified from rabbit, rat and human liver (Hudgin *et al.* 1974; Schwartz *et al.* 1981 *b*). Recent studies have begun to elucidate the characteristics of receptor-mediated endocytosis in this system. Using electron microscopic techniques Hubbard and colleagues have demonstrated the uptake of galactose-terminal glycoproteins by rat hepatic parenchymal cells and followed their subsequent transfer to a series of endocytic vesicles and ultimately to lysosomes (Wall & Hubbard 1981; Zeitlin & Hubbard 1982). Biochemical studies by Tolleshaug *et al.* (1977), Steer & Ashwell (1980) and others have provided evidence for a receptor-mediated uptake of asialoglycoproteins by isolated rat hepatocytes.

Using a combined biochemical and immunocytochemical electron micrographic approach, we have accumulated strong evidence for active recycling of the ASGP receptor and we have precisely localized the intracellular compartment where the dissociation of ASGP from its receptor occurs.

RESULTS AND DISCUSSION

We have performed the biochemical dissection of the pathway for ASGP uptake and degradation in the human hepatoma cell line Hep G2 isolated by Knowles *et al.* (1980). As seen in table 1, Hep G2 cells specifically bind [^{125}I]ASOR.† Such binding studies were per-

† ASOR, asialoorosomucoid.

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formed at 4 °C in order to minimize internalization of the ligand. Binding requires the presence of Ca^{2+} and is not substantially effected by the presence of a 100-fold excess (by mass) of orosomucoid or asialoagalactoorosomucoid. Pretreatment of the cells with neuraminidase renders them incapable of binding [^{125}I]ASOR. There are 150000–200000 high-affinity ASOR-binding sites per cell surface. These data are all consistent with the characteristics of

TABLE 2. SPECIFICITY OF RELEASE OF SURFACE-BOUND [^{125}I]ASOR FROM HEPATOMA CELLS

(Dishes were washed and incubated with [^{125}I]ASOR (2 μg ml^{-1}) in the standard manner (2 h, 4 °C). After washing in p.b.s. containing 1.5 mM $CaCl_2$, the indicated additions were made for the indicated time at 4 °C. Thereafter, one further rinse in p.b.s. with $CaCl_2$ was performed and the samples counted. Results are expressed as a percentage of the total [^{125}I]ASOR bound compared with the control ('none'). (In part adapted from Schwartz *et al.* (1981*a*).)

addition (concentration) (time)	[^{125}I]ASOR bound (%)
none	100
EDTA (5 mM) (3 min)	12
N-acetyl-galactosamine	
(100 mM) (10 min)	11
(50 mM) (10 min)	24
galactose (100 mM) (10 min)	62
N-acetyl-glucosamine (100 mM) (10 min)	100
ASOR (200 μg ml^{-1}) (300 min)	95

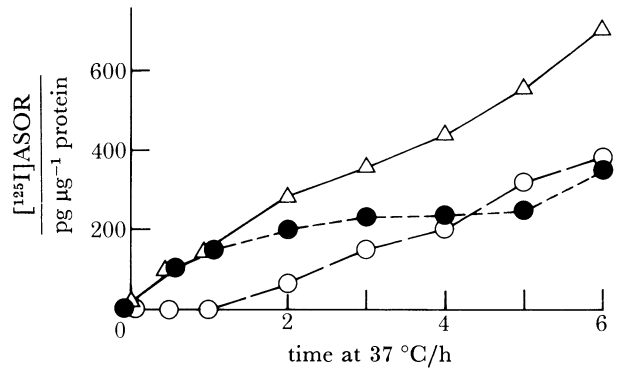

FIGURE 1. Uptake and degradation of [^{125}I]ASOR in human hepatoma cells at 37 °C. Tissue culture dishes containing 10^6 cells were washed and incubated with [^{125}I]ASOR (2 μg ml^{-1}) for various times at 37 °C. At the appropriate times the media were removed and analysed for ^{125}I degradation products. In addition, the cells were rinsed and analysed for cell-associated [^{125}I]ASOR. The figures represent the mean and range of duplicate determinations of cell-associated radioactivity (●), ^{125}I-degradation products of the media (○) and the sum of the two previous values (△). (Adapted from Schwartz *et al.* (1982).)

the asialoglycoprotein receptor in rat hepatocytes (Ashwell & Harford 1982). In addition, once bound to its receptor, [^{125}I]ASOR could be readily displaced by either a brief treatment at 4 °C with EDTA or *N*-acetylgalactosamine but only minimally by galactose. *N*-Acetyl-glucosamine or ASOR was without effect (table 2). The sensitivity of surface-bound [^{125}I]ASOR to displacement by EDTA or *N*-acetylgalactosamine provides a sensitive and convenient assay for surface-bound ligand; internalized ligand is resistant to such treatments.

At 37 °C, there is a linear increase in the amount of cell-associated [^{125}I]ASOR during the first 2 h (figure 1). A constant level of cell-associated ligand is reached by 2 h. There is little

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^{125}I label in degradation products in the medium before 1 h, and the linear increase in ^{125}I-degradation products begins by the second hour (figure 1). As expected, there is no detectable degradation of [^{125}I]ASOR when maintained for 6 h at 37 °C under identical conditions but in the absence of cells (data not shown). As measured by the sum of cell-associated and degraded ^{125}I radioactivity, the overall rate of cellular uptake of ASOR is constant at 0.02–0.03 pmol min^{-1} per 10^6 cells for at least 6 h (figure 1). In 28 independent experiments the rate of [^{125}I]ASOR uptake at a concentration of 2 μg ml^{-1} at 37 °C, assessed over the first 60 min, averaged 0.029 ± 0.001 pmol min^{-1} per 10^6 cells, as we have reported earlier (Schwartz *et al.* 1981*a*, 1982).

TABLE 3. SURFACE RECEPTOR DISTRIBUTION AND UPTAKE OF [^{125}I]ASOR IN HEPATOMA CELLS AFTER TRYPSIN TREATMENT

(Dishes containing monolayer cells were rinsed and incubated with [^{125}I]ASOR (2 μg ml^{-1}) at either 4 °C (specific binding) or 37 °C (uptake) (Schwartz *et al.* 1981*a*). Cell suspensions prepared with either EDTA or EDTA/trypsin were incubated with [^{125}I]ASOR (2 μg ml^{-1}) at either 4 °C (specific binding) or 37 °C (uptake). In addition, samples were incubated with cycloheximide (CLX) (100 μg ml^{-1}) with or without an additional 60 min at 37 °C with ASOR. Results (means ± s.e.) are presented of duplicate (binding) or triplicate (uptake) values. Initially, a fraction x of the functional receptor is internal to the cell, and $(1-x)$ is on the surface. After trypsin treatment there is a residual fraction, T, of surface receptors remaining. Thus, the total remaining fraction of cell-associated receptors is equal to the rate of uptake of [^{125}I]ASOR, relative to control cells. Solution of this equation yields the value of x, the fraction of receptors internal to the cell. (Adapted in part from Schwartz *et al.* (1982).)

	treatment	CLX	specific binding pmol/10^6 cells	rate of [^{125}I]ASOR uptake (60 min) pmol/(10^6 cells/min)	receptor on the cell surface (%)
(A)	monolayer–EDTA	–	0.29 ± 0.04	0.023 ± 0.001	
	suspension–EDTA	–	0.33 ± 0.04	0.023 ± 0.001	
	suspension–EDTA/trypsin	–	0.02 ± 0.00	0.004 ± 0.000	87.9
(B)	suspension–EDTA/trypsin + ASOR	–	0.04 ± 0.00†	(0.004)	—
	suspension–EDTA/trypsin + ASOR	+	0.05 ± 0.01†	(0.004)	75

† These samples were 'stripped' of surface-bound ASOR with EDTA before determination of specific binding with [^{125}I]ASOR.

Because the rate of ligand uptake (ligand flux) is dependent upon the total cell complement of functional receptors that participate in this process, we have determined the cell receptor distribution by destroying cell-surface receptors with protease. As shown in table 3, single-cell suspensions of Hep G2, prepared by treatment of monolayer cultures with an EDTA solution at 4 °C, bind at 4 °C the same amount of [^{125}I]ASOR as do cells assessed under standard conditions in monolayer culture. The rate of ligand uptake at 37 °C is also unimpaired. However, if trypsin is included in the EDTA solution at 4 °C (as is used for dispersing monolayer cells), binding of [^{125}I]ASOR to cells is inhibited by over 90 %, indicating that virtually all surface receptors are destroyed by this protease. When trypsin-treated cells are incubated at 37 °C, uptake of [^{125}I]ASOR is linear with time (data not shown) but is only 20 ± 2 % of that of control cells. Taking into account that only 94 % of the surface receptors is actually destroyed by trypsin, the data in table 3 indicate that in growing Hep G2 cells approximately 88 % of the functional receptor is on the surface, and 12 % is internal. When cells that had been trypsinized were then exposed to cycloheximide to prevent further protein synthesis, and allowed to incubate at 37 °C but in the presence of unlabelled ASOR (2 μg ml^{-1})

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for 60 min, the remaining functional receptors redistributed themselves such that approximately 75 % were again on the cell surface and 25 % internal (table 3). Thus, during steady-state ligand binding and internalization, more receptor is internal than in growing cells.

Because the uptake and degradation of ligand continues at a steady rate of 15000 molecules per cell per minute independent of new receptor synthesis (Schwartz *et al.* 1982) for at least 6 h and because there are 150000–200000 binding sites per cell surface, either there must exist a large pool of previously synthesized receptor within the cell, or receptor reuse must occur to some extent. If no reuse occurs, then the functional receptor pool within the cell must be at least 30–60-fold greater than the number of surface receptors. However, in these Hep G2 cells, 86 % of all functional receptors are on the cell surface. As calculated from the total number of functional receptors per cell (225000) and the rate of ligand uptake (15000 molecules per minute at an ASOR concentration of 2 μg ml^{-1}), each receptor must recycle the ligand, on the average, every 15.9 min ($= 225000 \div 15000$ min^{-1}). These observations and calculated values were all obtained at a ligand concentration of 50 nM (2 μg ml^{-1}). Obviously, at higher ligand concentrations the total cycle time will decrease as will the time required for ligand binding, until a point is reached at which binding is no longer rate-limiting. The rate of ligand uptake and degradation at 10–20 μg of [^{125}I]ASOR ml^{-1} (i.e. 30000 per cell per minute) is double that at 2 μg ml^{-1} (Schwartz *et al.* 1982), and the cycle time at 10–20 μg ml^{-1} is about half that at 2 μg ml^{-1}, or 7.9 min.

At 2 μg ml^{-1} ASOR, binding of ligand to surface receptors requires a mean time of 8.7 min. Internalization of receptor–ligand complexes requires a mean of 2.2 min, whereas a mean of 4.2 min is required for the internalized receptor to dissociate its ligand and return to the cell surface (Schwartz *et al.* 1982). Each of these rate constants was determined by two or more independent means; the sum of these times yields 15.1 min for the total cycle time of the asialoglycoprotein receptor.

ASGP ligands are therefore capable of being taken up and processed through to the lysosomes at a considerable rate (see also Ashwell & Harford 1982), whereas the receptor is apparently spared degradation. Additional biochemical studies have demonstrated that the intracellular half life of ASGP ligand taken up by receptor-mediated endocytosis is about 15–20 min, whereas that of the receptor is probably greater than 40 h (see Ashwell & Harford 1982).

Importantly, in Hep G2 cells, degradation of internalized ligand begins only after 20–30 min, a time much longer than the total cycle time of the receptor. Such studies suggest that receptor is not transferred to lysosomes, a conclusion substantiated by our morphological studies.

We have used our recently developed double-labelling immunocytochemical electron microscopic technique, with antibodies against both ASGP ligand and ASGP receptor, to visualize the compartment in which dissociation of the ligand–receptor complex occurs. Asialofetuin (1–6 mg) was administered in 1 ml physiological saline containing 1.5 mM $CaCl_2$ to adult rats by continuous infusion into a tail vein over 30–60 min, followed by perfusion fixation with 2 % formaldehyde – 0.5 % glutaraldehyde. Cryosectioning and immunolabelling with colloidal gold adsorbed to staphylococcal protein A were essentially as described previously (Geuze *et al.* 1981, 1982*a*). Affinity-purified monospecific rabbit antibodies against the purified rat liver ASGP receptor (Schwartz *et al.* 1981*b*) and against purified asialofetuin were employed.

Both ligand and receptor are taken up from the sinusoidal cell surface in clathrin-coated vesicles, which deliver the complexes to vesiculotubular structures (Geuze *et al.* 1982*b*). Both

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receptor and ligand were found associated with the membrane of small clathrin-coated vesicles close to the cell surface. Little or no free ligand occurred within the lumen of these vesicles. We also identified other larger vesicles found at some distance from the plasma membrane, which contain ligand accumulated within the lumen. The membranes of these latter vesicles contained little receptor, but receptor was concentrated in detached tubular extensions that were largely free of ligand (figure 2, plate 1). No significant receptor labelling was ever found within the vesicle lumen. Interestingly, receptor was not uniformly distributed along the membrane of these larger vesicles, but was either dispersed in clusters along the vesicle membrane or appeared as accumulations at the poles, where vesicles and thin membranous tubules approximated each other or were continuous. In most such vesicles, receptor labelling was either low compared with the connected or adjacent tubules, or was absent. The intensity of ligand labelling was greatest in larger vesicles, which also showed the lowest receptor labelling.

In the bile capillary region the larger vesicles often contain smaller internal vesicles. These multivesicular bodies lacked receptor but contained abundant ligand in the matrix space outside the internal vesicles.

Therefore, once internalized in coated vesicles, ligand accumulated in vesicles ranging in diameter from 0.2 μm at the sinusoidal cell surface to about 0.8 μm in the lysosomal area. The amount of ligand accumulated and the diameter of the vesicle increased in parallel. In the smaller, more peripheral, vesicles, ligand was membrane-associated, presumably with the membrane-bound receptor that was present in abundance in these vesicles. A diffuse membrane distribution of receptor was seen in those vesicles with receptor associated at ligand. In the vesicles with ligand free in the lumen, receptor was concentrated heavily at those sites of the vesicles at which thin, membranous tubules with intense receptor labelling were connected. This double-labelling pattern strongly suggests that these curl-tailed vesicles represent the compartment of uncoupling of receptor and ligand. We have suggested the acronym CURL to identify this compartment of dissociation (Geuze *et al.* 1982*b*). We believe that these tubules may represent an intermediate in the recycling of the receptor to the plasma membrane.

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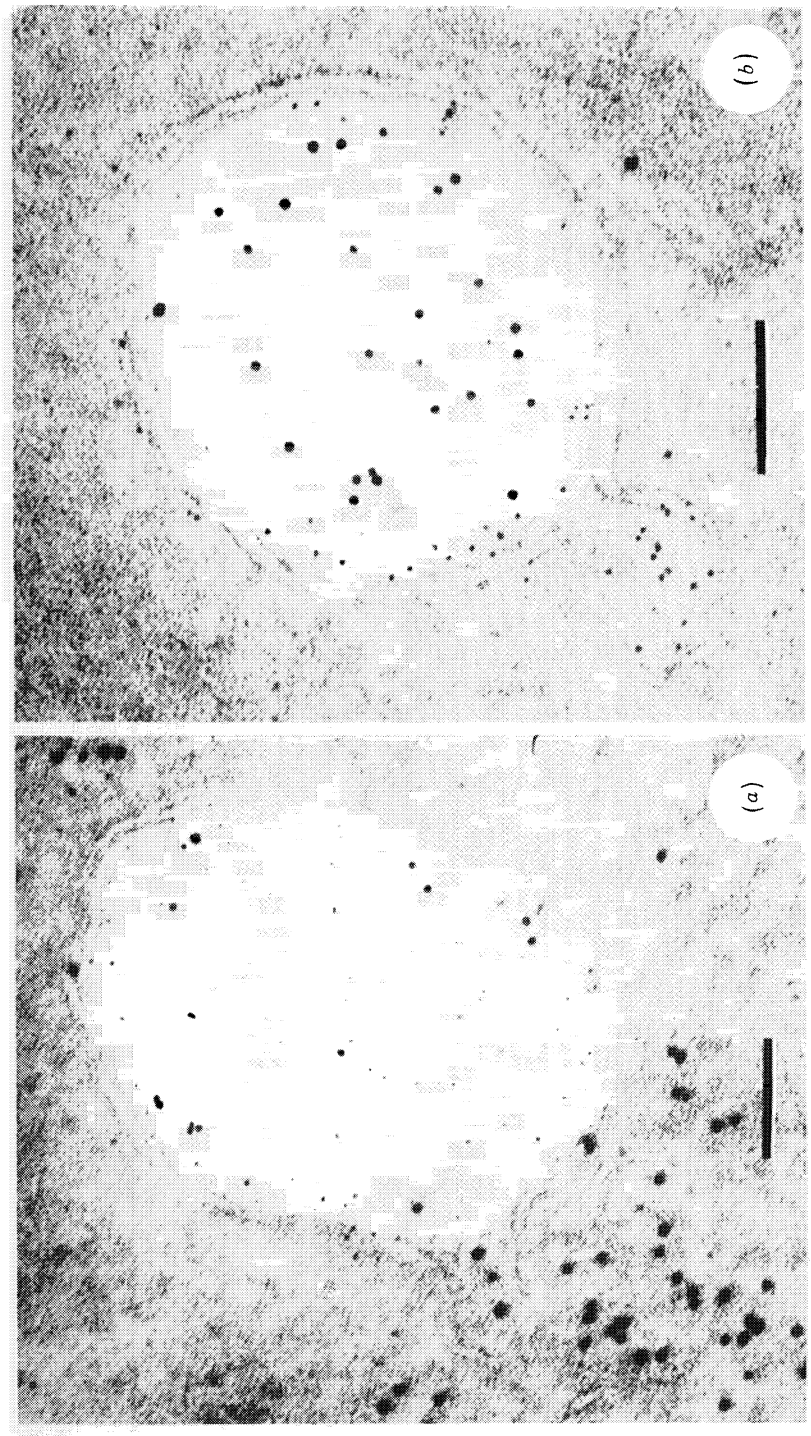

FIGURE 2. (*a*) Immunocytochemical electron micrograph of ultrathin cryosections from perfusion-fixed rat liver during continuous infusion of asialofetuin. Ligand was labelled first with anti-ASF antibody and then with 5 nm colloidal gold–protein A. Thereafter ASGP receptor was immunolabelled with antibody and then with 8 nm colloidal gold–protein A. Free ligand can be seen in the lumen of the vesicular portion of this sorting vesicle, which also shows scarce and heterogeneous receptor distribution. Receptor labelling is intense over the connecting tubules. Bar = 0.1 μm. (*b*) Similar to (*a*) except that receptor is labelled with 5 nm gold whereas ligand is labelled with 8 nm gold. Receptor is located predominantly at the fold where a tubule with heavy receptor labelling is connected. Most of the ligand is present free within the vesicle lumen. Bar = 0.1 μm.

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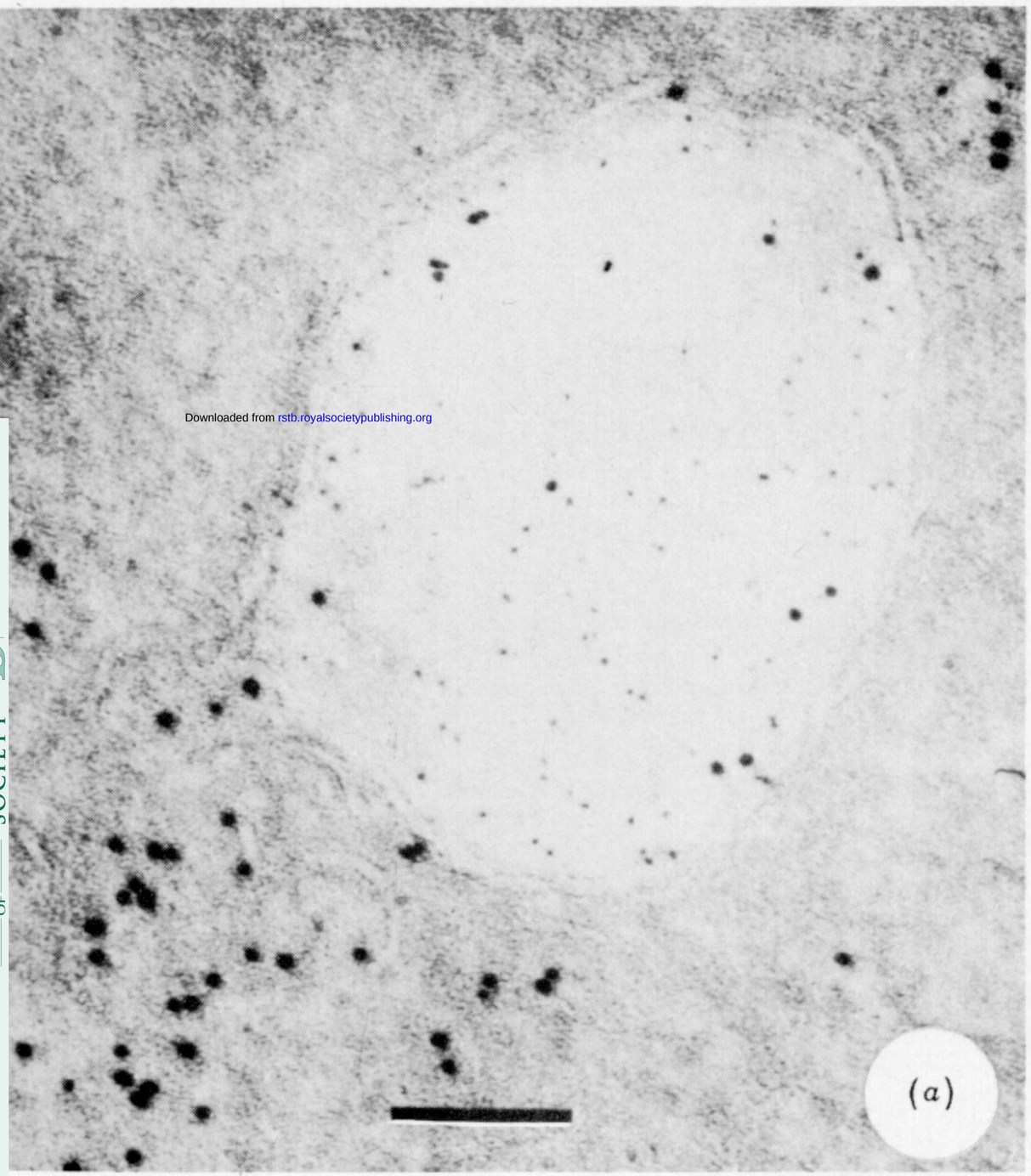

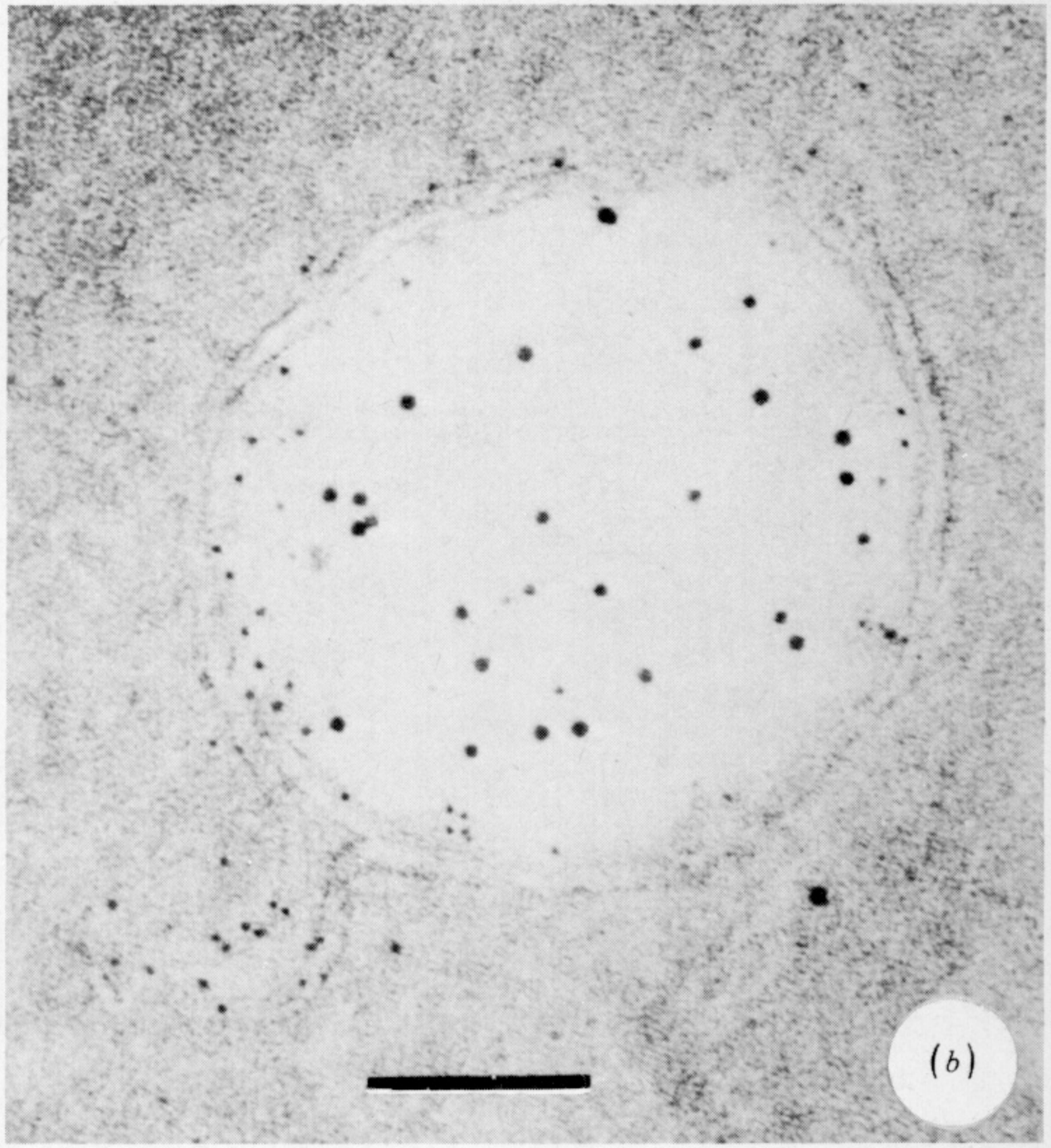

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