STUDIES ON THE INTERNALIZATION AND INTRACELLULAR TRANSPORT OF HORSERADISH PEROXIDASE IN CHINESE HAMSTER OVARY CELLS

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(ABSTRACT)

Soluble horseradish peroxidase (HRP) is internalized by Chinese hamster ovary cells, a cell line of fibroblastic origin (Adams et al., 1982). We have confirmed this result by showing no inhibition of uptake in the presence of divalent cation chelators (EGTA Mg or EDTA), excess (19 mg/ml) yeast mannan (an inhibitor of uptake through a mannose/N-acetylglucosamine receptor) or using periodate treated HRP. Periodate treatment destroys the ring structure of sugars on HRP which have hydroxyl groups on adjacent ring carbons, eliminating sugar mediated uptake of HRP. Once internalized, HRP is found in endocytic vesicles which by HRP-cytochemical staining, show deposits which rim the luminal face of vesicle

membrane. Once HRP is in lysosomes, cytochemical deposits are luminal. To test if HRP is actually associated with vesicle membrane, a hypotonic lysis assay was used. Postnuclear supernatants (PNS) from cells pulse labeled with HRP were lysed and the percent of HRP sedimenting with a high speed membrane fraction was used as a measure of membrane association. After a pulse, >60% of the total HRP internalized was pelletable. Hypotonic lysis of a PNS at different pH and temperature showed no significant difference in "pelletability" from 4°C to 37°C at neutral pH and only a slight decrease in "pelletability" with increased temperature (4°C to 37°C) at pH 4.6. Binding of HRP in a membrane preparation was pH and temperature stable. Uptake of native HRP in the presence of yeast mannan (19 mg/ml) or using periodate treated HRP also had little effect on "pelletability", suggesting the absence of sugar specific binding in endocytic vesicles. Using the hypotonic lysis assay of a PNS after different chase times, HRP dissociation from membrane was observed over a 30 minute chase period. Internalized HRP in the presence of yeast mannan (19 mg/ml), intravesicular pH elevators HEPES (40 mM) or monensin (10 μM), or substances which should deplete cellular ATP NaF/KCN (2 mM / 1 mM), showed no inhibition of dissociation kinetics. A chase at 17°C inhibited dissociation of HRP over the entire 30 minute

period. This HRP binding site(s) appears unique to endocytic vesicles.

A minimum of four steps in transport have been identified based on their sensitivity to inhibitors. HRP transport, identified by Percoll density gradient fractionaton, was inhibited at 17°C and was sensitive to pH elevators (NH_4Cl, monensin, HEPES) and ATP depletion (NaF/KCN). Inhibition of transport appeared to be independent of HRP dissociation except at early temperature sensitive step(s) These results suggest that transport inhibition may be due to an effect on a) inhibition of membrane dissociation (early step(s)) and alteration of membrane fluidity (later steps) by reduced temperature and b) transmembrane events by pH elevators and ATP depletion.

DEDICATION

This is dedicated to my dad who helped instill in me a sense of perseverance and compassion. He always supported me in my endeavors and had faith in my efforts. Without him, this work would never have been possible however, he died before seeing its completion. This degree is as much a part of him as it is a part of me.

I thank you for your love and your helping hand.

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Chapter I

General Introduction

Endocytosis in eukaryotic cells may be described as the uptake of extracellular material into membrane-limited vesicles in the cytosol. Internalized material may include extracellular fluid, dissolved solutes, particles (including whole cells) or macromolecules (for review see Steinman et al., 1983). One of the first descriptions of endocytosis was by Metchnikoff (1893). He reported the uptake of yeast particles by cells of the water flea. Later Lewis (1931) described the uptake of extracellular fluid by mammalian cells. However not until 1963 was the term "endocytosis" introduced by de Duve (1963).

Endocytosis can be divided into phagocytosis and pinocytosis. Phagocytosis may be thought of as "cell eating", a process responsible for the internalization of large (0.5 - > 1 μM) particles such as yeast cells (e.g., Ryter and de Chastellier, 1977; Bowers, 1980; Bowers et al., 1981), bacteria (e.g., Michell et al., 1969; Griffin and Silverstein, 1974), red blood cells (e.g., North, 1966; Rabinovitch, 1969), latex spheres (e.g., Githens and Karnovsky, 1973; Hubbard and Cohn, 1975) and paraffin-oil emulsions (e.g., Stossel et al., 1972). Phagocytosis is generally associated with certain specialized cell types

like macrophages or neutrophils however, evidence shows that it can also occur in fibroblasts (e.g., Hubbard and Cohn, 1975; Storrie and Chadwick, 1980). Phagocytosis is an inducible process and occurs after binding of a particle to the plasma membrane (for review see Silverstein et al., 1977). However, it should be noted that particle binding alone is not sufficient for phagocytosis (Jones and Hirsch, 1971; Bianco et al., 1975; for review see Silverstein et al., 1977). Uptake by phagocytosis is energy dependent (for review see Silverstein et al., 1977; Besterman and Low, 1983), requires divalent cations (e.g., Warr, 1980; Ito et al., 1981) and is limited in the total amount of uptake which may occur at any one time (e.g., Michell et al., 1969; Werb and Cohn, 1972; Leijh et al., 1979). Phagocytosis can result in the internalization of up to 50% of the total plasma membrane (Werb and Cohn, 1972; for review see Silverstein et al., 1977) without a decrease in cell volume.

Pinocytosis results in the uptake of a wide range of macromolecules and extracellular fluid. This process can be divided into adsorptive and fluid phase endocytosis. Adsorptive endocytosis may be further divided based on whether material binds specifically or non-specifically to plasma membrane prior to uptake. Non-specific binding is

seen with cationic ferritin for example, which binds to many anionic sites on cell surfaces (for review see Besterman and Low, 1983). Specific binding to plasma membrane and subsequent internalization is referred to as receptor-mediated endocytosis. Molecules (ligands) entering cells this way constitute a physiologically important set of substances which include epidermal growth factor (Carpenter and Cohn, 1976), transferrin (Jandl and Katz, 1963), insulin (Kahn, 1976) and low density lipoprotein (Anderson et al., 1976; for review see Goldstein and Brown, 1977) as well as Semliki forest virus (Dales, 1973; Helenius et al., 1980), cholera toxin (Van Leuven et al., 1978, 1979; Neville and Chang, 1978) and α-2-macroglobulin-protease complexes (Kaplan and Nielsen, 1979). In the absence of ligand, cell surface receptors may be distributed either diffusely (e.g., Maxfield et al., 1978; for review see Pastan and Willingham, 1985) or clustered into small areas called coated pits (e.g., Griffin and Griffin, 1980; Anderson et al., 1982; Zeitlin and Hubbard, 1982). Binding of ligand to diffuse receptors results in a receptor-ligand complex accumulating in coated pits, concentrating ligand in these areas. Coated pits are small indentations in the plasma membrane which have a thick protein coat on the cytoplasmic surface. The coated pits are thought to infold

and pinch off, forming coated vesicles (for review see Pearse and Bretscher, 1981) which quickly lose their coats (e.g., Thrasher et al.,1973; Van Oss, 1978; Griffin and Griffin, 1980) forming a smooth endocytic vesicle.

Coated vesicles were first observed by Roth and Porter (1964) and were first isolated by Pearse (1975) apparently free of plasma membrane. The coat material of these vesicles, as well as the coated pits, is mainly composed of clathrin, a 180 kDa protein (Pearse, 1975; Pastan and Willingham, 1981; Pearse and Bretscher, 1981). Clathrin has also been found on the cytoplasmic side of some areas of the Golgi apparatus. Recent evidence shows that the loss of clathrin from coated structures in endocytic pathways occurs in less than a minute and likely in less than 20 seconds (for review see Pastan and Willingham, 1985). Coated vesicles also appear to have an ATP-dependent proton pump (Forgac et al., 1983; Van Dyke et al., 1984) although a functional role for this pump in these vesicles has not been established.

Evidence for a coated vesicle which is independent from plasma membrane, has been disputed by Willingham and Pastan (Willingham and Pastan, 1980; Pastan and Willingham, 1981) who present evidence that coated 'vesicles' remain tethered to the plasma membrane by a thin neck and that endocytic vesicles form by a budding of

smooth membrane from the coated pit. Results from studies using serial sections of coated pits and vesicles are conflicting (Petersen and Van Deurs, 1983; Willingham and Pastan, 1983). The transient role of these coated structures in intracellular events in endocytic pathways makes any such structure a minor intracellular population.

Smooth endocytic vesicles are known to be acidic and appear to possess an ATP-dependent proton pump (Maxfield, 1982; Tycko and Maxfield, 1982; Galloway et al., 1983; Yamashiro et al., 1983). This has been proposed as a means for ligand-receptor dissociation, in a number of systems and is based on evidence which shows a decreased affinity of ligand for receptor at a mildly acidic (< 6.0) pH (Pricer and Ashwell, 1971; Gonzalez-Noriega et al., 1980; Sahagian et al., 1981; Maxfield, 1982; Tietze et al., 1982). Separation of ligand and receptor would allow intracellular sorting of the two into separate compartments having different destinations. Such a segregation of ligand and receptor has been demonstrated by Geuze et al. (1983) in the asialoglycoprotein receptor system to occur in a compartment of uncoupling of receptor and ligand (CURL). The receptor in this system (for review see Ashwell and Harford, 1982; Schwartz et al., 1984; Oka and Weigel, 1983), as well as in the low density lipoprotein system (Anderson et al., 1982), has been shown

to recycle back to the cell surface while ligand is transported to lysosomes where it is degraded (for review see Steinman et al., 1983). Variations of this pathway do occur. With the macrophage Fc receptor (Mellman et al., 1983) and acetylcholine receptor (Gardner and Fambrough, 1977) no dissociation of ligand from receptor occurs and both ligand and receptor are transported to lysosomes and degraded. In still other cases, neither receptor nor ligand are degraded. Transferrin and its receptor recycle intact back to the plasma membrane (for review see Hanover and Dickson, 1985). In intestinal epithelial cells, IgG is transported from the lumen of the intestine to the opposing cell surface and released (Abrahamson and Rodewald, 1981). In the case of epidermal growth factor (Das and Fox, 1978; Dunn and Hubbard, 1984) and insulin (Kasuga et al., 1981; Marshall et al., 1981; Fehlmann et al., 1982) results for and against receptor recycling have been presented.

In a fluid phase uptake system, Friend and Farquhar (1967) observed HRP, a solute, in coated vesicles. Pastan and Willingham (1985) also found HRP localized in coated structures. Work by Marsh and Helenius (Marsh and Helenius, 1980; Helenius and Marsh, 1982) indicate that the fluid volume occupied by virions internalized by receptor-mediated endocytosis is equal to observed

decreases in solute internalization (fluid phase endocytosis). Similar conclusions were reached by Ryser et al. (1982) through a different approach. HRP internalization is almost certainly "piggy backed" on a coated pit pathway (for review see Pastan and Willingham, 1985). In this dissertation, HRP is the endocytic marker used.

The internalized material present in endocytic vesicles is transported to lysosomes in 5-15 minutes (Steinman et al., 1976; Storrie et al., 1984). This transport occurs through endocytic vesicles which vary in density and are capable of being diserned from one another by isopycnic centrifugation (Merion and Sly, 1983; Storrie et al., 1984). In cell types with well developed polarity such as hepatocytes, differences in morphology and subcellular localization can be observed between various classes of endocytic vesicles (Helenius et al., 1983).

A large amount of fluid and plasma membrane are internalized during endocytosis. Work by Steinman et al., (1976) showed in macrophages and L cells that a cell surface equivalent of membrane was internalized every 33 and 125 minutes respectively while the size of the endocytic vesicle and lysosomal compartments remained constant. From these data, it was suggested that recycling of membrane back to the cell surface must occur.

Since that time, a number of studies have demonstrated a large degree of exocytosis of fluid and recycling of membrane, pointing out the importance of exocytosis in the endocytic process (e.g., Besterman et al., 1981; Storrie et al., 1981; Adams et al., 1982; Daukas et al., 1983).

The large amount of membrane flow into a cell during the process of endocytosis, has stimulated interest as to the composition of internalized membrane. Work using macrophages and a lactoperoxidase radioiodination system has shown that vesicle membrane is representative of the plasma membrane (Mellman et al., 1980). However, Taylor et al. (1971), using antibodies to surface markers on lymphocytes, reported a selective internalization of some membrane proteins. In addition, a redistribution of surface proteins, analogous to a receptor system, should result in a selective uptake of some surface proteins. Bretscher et al. (1980) found at least two major cell surface proteins excluded from coated pits. These conflicting results may arise from differences in the methods used, as suggested by Besterman and Low (1983).

Endocytosis in mammalian cells is a constitutive process which is temperature sensitive and requires energy (for review, see Silverstein et al., 1977). It appears independent of microtubule disruption with colchicine (Pesanti and Axline, 1975), while results regarding the

involvment of microfilaments are conflicting (e.g., Wagner et al., 1971; Wills et al., 1972). Endocytosis appears to be regulated by a number of extracellular factors such as serum, phorbol myristate acetate and cell density. These have caused increases in the rate of accumulation of endocytic markers. Apparent increases in endocytosis may be the result of an increased kinetics of the internalization process. Alternatively, elevated accumulation of material in the cell may be due to a decrease in exocytosis of internalized material (Besterman et al., 1983; Swanson et al., 1985).

The precise mechanisms of transport to lysosomes of internalized material remain elusive. The extent and nature of processing of internalized material and of endocytic vesicles is currently unknown. Details of intracellular transport processess and the underlying mechanisms are important to an understanding of basic cellular function. This study was undertaken to expand the present knowledge of intracellular transport in a fluid phase system using a mammalian cell line.

References

Abrahamson, D.R., Rodewald, R. (1981) Evidence for the sorting of endocytic vesicle contents during the receptor-mediated transport of IgG across the newborn rat intestine. J. Cell Biol., 91:270-280.

Adams, C.J., Maurey, K.M., Storrie, B. (1982) Exocytosis of pinocytic contents by Chinese hamster ovary cells. J. Cell Biol., 93:632-637.

Anderson, R.G.W., Brown, M.S., Beisiegel, U., Goldstein, J.L. (1982) Surface distribution and recycling of the low density lipoprotein receptor as visualized with antireceptor antibodies. J. Cell Biol., 93:523-531.

Anderson, R.G.W., Goldstein, J.L., Brown, M.S. (1976) Localization of low density lipoprotein receptors on plasma membrane of normal human fibroblasts and their absence in cells from a familial hypercholesterolemia homozygote. Proc. Natl. Acad. Sci. U.S.A., 73:2434-2438.

Ashwell, G. and Harford, J. (1982) Carbohydrate-specific receptors of the liver. Ann. Rev. Biochem., 51:531-534.

Besterman, J.M., Airhart, J.A., Low, R.B., Rannels, D.E. (1983) Pinocytosis and intracellular degradation of exogenous protein: Modulation by amino acids. 96:1586-1591.

Besterman, J.M., Airhart, J.A., Woodworth, R.C., Low, R.B. (1981) Exocytosis of pinocytosed fluid in cultured cells: Kinetic evidence for rapid turnover and compartmentation. J. Cell Biol., 91:716-727.

Besterman, J.M. and Low, R.B. (1983) Endocytosis: A review of mechanisms and plasma membrane dynamics. Biochem. J., 210:1-13.

Bianco, C., Griffin, F.M.Jr., Silverstein, S.C. (1975) Studies of the macrophage complement receptor. Alteration of receptor function upon macrophage activation. J. Exp. Med., 141:1278-1290.

Bowers, B. (1980) A morphological study of the plasma and phagosome membranes during endocytosis. J. Cell Biol., 84:246-260.

Bowers, B., Olszewski, T.E., Hyde, J. (1981) Morphometric analysis of volumes and surface areas in membrane

compartments during endocytosis in Acanthamoeba. J. Cell Biol., 88:509-515.

Bretscher, M.S., Thomson, J.N., Pearse, B.M.F. (1980) Coated pits act as molecular filters. Proc. Natl. Acad. Sci. U.S.A., 77:4156-4159.

Carpenter, G. and Cohen, S. (1976) ^{125}I-labeled human epidermal growth factor. Binding, internalization, and degradation in human fibroblasts. J. Cell Biol., 71:159-171.

Dales, S. (1973) Early events in cell-animal virus interactions. Bacteriol. Rev., 37:103-135.

Das, M. and Fox, C.F. (1978) Molecular mechanism of mitogen action: Processing of receptor induced by epidermal growth factor. Proc. Natl. Acad. Sci. U.S.A., 75:2644-2648.

Daukas, G., Lauffenburger, D.A., Zigmond, S. (1983) Reversible pinocytosis in polymorphonuclear leukocytes. J. Cell Biol., 96:1642-1650.

De Duve, C. (1963) Lysosomes. Ciba Found. Symp. London, J.& A. Churchill, Ltd.

Dunn, W.A. and Hubbard, A.L. (1984) Receptor-mediated endocytosis of epidermal growth factor by hepatocytes in the perfused rat liver: Ligand and receptor dynamics. J. Cell Biol., 98:2148-2159.

Fehlmann, M., Carpentier, J.- L., Van Obberghen, E., Freychet, P., Thamm, P., Saunders, D., Brandenburg, D., Orci, L. (1982) Internalized insulin receptors are recycled to the cell surface in rat hepatocytes. Proc. Natl. Acad. Sci. U.S.A., 79:5921-5925.

Forgac, M., Cantley, L., Wiedenmann, B., Altstiel, L., Branton, D. (1983) Clathrin-coated vesicles contain an ATP-dependent proton pump. Proc. Natl. Acad. Sci. U.S.A., 80:1300-1303.

Friend, D.S. and Farquhar, M.G. (1967) Functions of coated vesicles during protein absorption in the rat vas deferens. J. Cell Biol., 35:357-378.

Galloway, C.J., Dean, G.E., Marsh, M., Rudnick, G., Mellman, I. (1983) Acidification of macrophage and

fibroblast endocytic vesicles in vitro. Proc. Natl. Acad. Sci. U.S.A., 80:3334-3338.

Gardner, J.M. and Fambrough, D.M. (1977) Acetylcholine receptor degradation measured by density labeling: Effects of cholinergic ligands and evidence against recycling. Cell, 16:661-674.

Geuze, H.J., Slot, J.W., Stros, G.J.A.M., Lodish, H.F., Schwartz, A.L. (1983) Intracellular site of asialoglycoprotein receptor-ligand uncoupling: Double-label immunoelectronmicroscopy during receptor mediated endocytosis. Cell, 32:277-287.

Githens, S. III and Karnovsky, M.L. (1973) Phagocytosis by the cellular slime mold Polysphondylium pallidum during growth and development. J. Cell Biol., 58:536-548.

Goldstein, J.L. and Brown, M.S. (1977) The low-density lipoprotein pathway and its relation to atherosclerosis. Ann. Rev. Biochem., 46:897-930.

Gonzalez-Noriega, A., Grubb, J.H., Talkad, V., Sly, W.S. (1980) Chloroquine inhibits lysosomal enzyme pinocytosis and enhances lysosomal enzyme secretion by impairing receptor recycling. J. Cell Biol., 85:839-852.

Griffin, F.M. and Griffin, J.A. (1980) Augmentation of macrophage complement receptor function in vitro II. Characterization of the effects of a unique lymphokine upon the phagocytic capabilities of macrophages. J. Immunol., 125:844-849.

Griffin, F.M.Jr. and Silverstein, S.C. (1974) Segmental response of the macrophage plasma membrane to a phagocytic stimulus. J. Exp. Med., 139:323-326.

Hanover, J.A. and Dickson, R.B. (1985) Transferrin: Receptor-mediated endocytosis and iron delivery. In: Endocytosis. I. Pastan and M.C. Willingham eds. Plenum Press, New York. pp. 131-161.

Helenius, A., Kartenbeck, J., Simons, K., Fries, E. (1980) On the entry of entry of Semliki forest virus into BHK-21 cells. J. Cell Biol., 84:404-420.

Helenius, A. and Marsh, M. (1982) Endocytosis of Enveloped Animal Viruses. Ciba Sym., 92:59-69.

Helenius, A., Mellman, I., Wall, D., Hubbard, A. (1983) Endosomes. TIBS, 8:245-250.

Hubbard, A.L. and Cohn, Z.A. (1975) Externally disposed plasma membrane proteins. II. Metabolic fate of iodinated polypeptides of mouse L cells. J. Cell Biol., 64:461-479.

Ito, T., Ueda, M.J., Okada, T.S., Ohnishi, S.I. (1981) Phagocytosis by macrophages. II. The dissociation of the attachment and ingestion steps. J. Cell Sci., 51:189-201.

Jandl, J.H. and Katz, J.H. (1963) The plasma-to-cell cycle of transferrin. J. Clin. Invest., 42:314-326.

Jones, T.C. and Hirsch, J.G. (1971) The interaction in vitro of mycoplasma pulmonis with mouse peritoneal macrophages and L-cells. J. Exp. Med., 133:231-259.

Kahn, C.R. (1976) Membrane receptors for hormones and neurotransmitters. J. Cell Biol., 70:261-286.

Kaplan, J. and Nielsen, M.L. (1979) Analysis of macrophage surface receptors. I. Binding of α_{-2}-macroglobulin-protease complexes to rabbit alveolar macrophages. J. Biol. Chem., 254:7323-7328.

Kasuga, M., Kahn, C.R., Hedo, J.A., Obberghen, E.V., Yamada, K.M. (1981) Insulin-induced receptor loss in cultured human lymphocytes is due to accelerated receptor degradation. Proc. Natl. Acad. Sci. U.S.A., 78:6917-6921.

Leijh, P.C.J., Van Den Barselaar, M.Th., Van Zwet, T.L., Dubbeldman-Rempt, I., Van Furth, R. (1979) Kinetics of phagocytosis of Staphylococcus aureus and Escherichia coli by human granulocytes. Immunology, 37:453-465.

Lewis, W.H. (1931) Pinocytosis. Bull. Johns Hopkins Hosp. 49:17-36.

Marsh, M. and Helenius, A. (1980) Adsorptive endocytosis of Semliki forest virus. J. Mol. Biol., 142:439-454.

Marshall, S., Green, A., Olefsky, J.M. (1981) Evidence for recycling of insulin receptors in isolated rat adipocytes. J. Biol. Chem., 256:11464-11470.

Maxfield, F.R. (1982) Weak bases and ionophores rapidly and reversibly raise the pH of endocytic vesicles in cultured mouse fibroblasts. J. Cell Biol., 95:676-681.

Maxfield, F.R., Schlessinger, J., Shechter, Y., Pastan, I., Willinham, M.C. (1978) Collection of insulin, EGF, and α_{-2}-macroglobulin in the same patches on the surface of cultured fibroblasts and common internalization. Cell, 14:805-810.

Mellman, I.S., Plutner, H., Steinman, R.M., Unkeless, J.C., Cohn, Z.A. (1983) Internalization and degradation of macrophage Fc receptors during receptor-mediated phagocytosis. J. Cell Biol., 96:887-895.

Mellman, I.S., Steinman, R.M., Unkeless, J.C., Cohn, Z.A. (1980) Selective iodination and polypeptide composition of pinocytic vesicles. J. Cell Biol., 86:712-722.

Merion, M. and Sly, W.S. (1983) The role of intermediate vesicles in the adsorptive endocytosis and transport of ligand to lysosomes by human fibroblasts. J. Cell Biol., 96:644-650.

Metchnikoff, E. (1893) In: Lectures on Comparative Pathology of Inflamation. Paul, Kegan, Trench, Trabner and Co., London.

Michell, R.H., Pancake, S.J., Noseworthy, J., Karnovsky, M.L. (1969) Measurement of rates of phagocytosis. The use of cellular monolayers. J. Cell Biol., 40:216-224.

Neville, D.M. and Chang, T.- M. (1978) Receptor-mediated protein transport into cells. Entry mechanisms for toxins, hormones, antibodies, viruses, lysosomal hydrolases, asialoglycoproteins, and carrier proteins. Curr. Top. Membr. Transp., 10:65-150.

North, R.J. (1966) The localization by electron microscopy of acid phosphatase activity in guinea pig macrophages. J. Ultrastructure Res., 16:96-108.

Oka, J.A. and Weigel, P.H. (1983) Recycling of the asialoglycoprotein receptor in isolated rat hepatocytes. J. Biol. Chem., 258:10253-10262.

Pastan, I. and Willingham, M.C. (1985) The pathway of endocytosis. In: Endocytosis. I. Pastan and M.C. Willingham eds. Plenum Press, New York. pp. 1-44.

Pastan, I. and Willingham, M.C. (1981) Journey to the center of the cell: Role of the receptosome. Science, 214:504-509.

Pearse, B.M.F. (1975) Coated vesicles from pig brain: Purification and biochemical characterization. J. Mol. Biol., 97:93-98.

Pearse, B.M.F. and Bretscher, M.S. (1981) Membrane recycling by coated vesicles. Ann. Rev. Biochem., 50:85-101.

Pesanti, E.L. and Axline, S.G. (1975) Colchicine effects on lysosomal enzyme induction and intracellular degradation in the cultivated macrophage. J. Exp. Med., 141:1030-1046.

Petersen, O.W. and Van Deurs, B. (1983) Serial-section analysis of coated pits and vesicles involved in adsorptive pinocytosis in cultured fibroblasts. J. Cell Biol., 96:277-281.

Pricer, W.E.Jr. and Ashwell, G. (1971) The binding of desialylated glycoproteins by plasma membrane of rat liver. J. Biol. Chem., 246:4825-4833.

Rabinovitch, M. (1969) Phagocytosis of modified erythrocytes by macrophages and L2 cells. Exp. Cell Res., 56:326-332.

Roth, T.F. and Porter, K.R. (1964) Yolk protein uptake in the oocyte of the mosquito _Aedes aegypti_ L. J. Cell Biol., 20:313-332.

Ryser, H.J.- P., Drummond, I., Shen, W.- C. (1982) The cellular uptake of horseradish peroxidase and its poly(lysine) conjugate by cultured fibroblasts is qualitatively similar despite a 900-fold difference in rate. J. Cell. Physiol., 113:167-178.

Ryter, A. and de Chastellier, C. (1977) Morphometric and cytochemical studies of _Dictostelium discoideum_ in vegetative phase. Digestive system and membrane turnover. J. Cell Biol., 75:200-217.

Sahagian, G.G., Distler, J., Jourdian, G.W. (1981) Characterization of a membrane-associated receptor from bovine liver that binds phosphomannosyl residues of bovine testicular β-galactosidase. Proc. Natl. Acad. Sci. U.S.A., 78:4289-4293.

Schwartz, A.L., Bolognesi, A., Fridovich, S.E. (1984) Recycling of the asialoglycoprotein receptor and the

effect of lysosomotropic amines in hepatoma cells. J. Cell Biol., 98:732-738.

Silverstein, S.C., Steinman, R.M., Cohn, Z.A. (1977) Endocytosis. Ann. Rev. Biochem., 46:669-722.

Steinman, R.M., Brodie, S.E., Cohn, Z.A. (1976) Membrane flow during pinocytosis. Stereologic analysis. J. Cell Biol., 68:665-687.

Steinman, R.M., Mellman, I.S., Muller, W.A., Cohn, Z.A. (1983) Endocytosis and the recycling of plasma membrane. J. Cell Biol., 96:1-27.

Storrie, B., Chadwick, R.T. (1980) Immunofluorescent evidence for a transient association of actin with phagosomes in BHK 21 cells. Cell Biol. Int. Rep., 4:873-879.

Storrie, B., Dreesen, T.D., Maurey, K.M. (1981) Rapid cell surface appearance of endocytic membrane proteins in Chinese hamster ovary cells. Mol. Cell. Biol., 1:261-268.

Storrie, B., Pool, R.R.Jr., Sachdeva, M., Maurey, K.M., Oliver, C. (1984) Evidence for both prelysosomal and lysosomal intermediates in endocytic pathways. J. Cell Biol., 98:108-115.

Stossel, T.P., Mason, R.J., Hartwig, J., Vaughan, M. (1972) Quantitative studies of phagocytosis by polymorphonuclear leukocytes: Use of emulsions to measure the initial rate of phagocytosis. J. Clin. Invest., 51:615-624.

Swanson, J.A., Yirinec, B.D., Silverstein, S.C. (1985) Phorbol esters and horseradish peroxidase stimulate pinocytosis and redirect the flow of pinocytosed fluid in macrophages. J. Cell Biol., 100:851-859.

Taylor, R.B., Duffus, W.P.H., Raff, M.C., Depetris, S. (1971) Redistribution and pinocytosis of lymphocyte surface immunoglibulin molecules induced by anti-immunoglobulin antibody. Nature, New Biol., 233:225-229.

Thrasher, S.G., Yoshida, T., Van Oss, C.J., Cohen, S., Rose, N.R. (1973) Alteration of macrophage interfacial tension by supernatants of antigen-activated lymphocyte cultures. J. Immunol., 110:321-326.

Tietze, C., Schlesinger, P., Stahl, P. (1982) Mannose-specific endocytosis receptor of alveolar macrophages: Demonstration of two functionally distinct intracellular pools of receptor and their roles in receptor recycling. J. Cell Biol., 92:417-424.

Tycko, B. and Maxfield, F.R. (1982) Rapid acidification of endocytic vesicles containing α_2-macroglobulin. Cell, 28:643-651.

Van Dyke, R.W., Steer, C.J., Scharschmidt, B.F. (1984) Clathrin-coated vesicles from rat liver: Enzymatic profile and characterization of ATP-dependent proton transport. Proc. Natl. Acad. Sci. U.S.A., 81:3108-3112.

Van Leuven, F., Cassiman, J.- J., Van Den Berghe, H. (1978) Uptake and degradation of α_2-macroglobulin-protease complexes in human cells in culture. Exp. Cell Res., 117:273-282.

Van Leuven, F., Cassiman, J.- J., Van Den Berghe, H. (1979) Demonstration of an α_2-macroglobulin receptor in human fibroblasts, absent in tumor-derived cell lines. J. Biol. Chem., 254:5155-5160.

Van Oss, C.J. (1978) Phagocytosis as a surface phenomenon. Ann. Rev. Microbiol., 32:19-39.

Wagner, R., Rosenberg, M., Estensen, R. (1971) Endocytosis in Chang liver cells. Quantitation by sucrose-^{3}H uptake and inhibition by cytochalasin B. J. Cell Biol., 50:804-817.

Warr, G.A. (1980) A macrophage receptor for (mannose/glucosamine)-glycoproteins of potential importance in phagocytic activity. Biochem. Biophys. Res. Commun., 93:737-745.

Werb, Z. and Cohn, Z.A. (1972) Plasma membrane synthesis in the macrophage following phagocytosis of polystyrene latex particles. J. Biol. Chem., 247:2439-2446.

Willingham, M.C. and Pastan, I. (1980) The receptosome: An intermediate organelle of receptor-mediated endocytosis in cultured fibroblasts. Cell, 21:67-77.

Willingham, M.C. and Pastan, I. (1983) Formation of receptosomes from plasma membrane coated pits during endocytosis: Analysis by serial sections with improved

membrane labeling and preservation techniques. Proc. Natl. Acad. Sci., 80:5617-5621.

Wills, E.J., Davies, P., Allison, A.C., Haswell, A.D. (1972) Cytochalasin B fails to inhibit pinocytosis by macrophages. Nature, New Biol., 240:58-60.

Yamashiro, D.J., Fluss, S.R., Maxfield, F.R. (1983) Acidification of endocytic vesicles by an ATP-dependent proton pump. J. Cell Biol., 97:929-934.

Zeitlin, P.L. and Hubbard, A.L. (1982) Cell surface distribution and intracellular fate of asialoglycoproteins: A morphological and biochemical study of isolated rat hepatocytes and monolayer cultures. J. Cell Biol., 92:634-647.

Chapter II

Membrane Association of Horseradish Peroxidase in Endocytic Vesicles of Chinese Hamster Ovary (CHO) Cells.

Introduction

Horseradish peroxidase (HRP), a glycoprotein, is used widely as a marker for endocytosis. The popularity of HRP is due to its high enzymatic activity, stability and ease of localization by standard cytochemical procedures. HRP can be internalized by either fluid phase (Steinman and Cohn, 1972; Steinman et al., 1974; Adams et al., 1982) or receptor-mediated endocytosis (Kaplan and Nielsen, 1978; Sung et al., 1983; Lang and de Chastellier, 1985). For receptor-positive cells such as macrophages, the mode of uptake is dependent upon the concentration of HRP; at 1 mg/ml HRP, greater than 80% of uptake is by fluid phase endocytosis while at 25 μg/ml almost 90% of HRP internalization is through receptor mediated endocytosis (Lang and de Chastellier, 1985). Receptor-mediated endocytosis of HRP involves a mannose/N-acetylglucosamine specific receptor (Baynes and Wold, 1976; Stockert et al., 1976; Rodman et al., 1978; Sung et al., 1983). Mannose/N-acetylglucosamine residues comprise over 80% of the carbohydrate portion of HRP (Clarke and Shannon, 1976; Welinder, 1979). For fibroblasts HRP uptake is by fluid

phase endocytosis; uptake of HRP is proportional to its concentration in the medium and is linear over a wide range of concentrations (Steinman et al., 1974; Adams et al., 1982). For Chinese hamster ovary (CHO) cells, a cell line of fibroblastic origin, HRP uptake is unaffected by the addition of a variety of monosaccharides or yeast mannan, commonly used to inhibit sugar specific uptake (Adams et al., 1982; Pool et al., 1983).

Once internalized, HRP is found in two classes of vesicles. Initially, HRP cytochemical deposits are localized in endocytic vesicles (a prelysosomal compartment) rimming the interior membrane surface (Steinman et al., 1974; Adams et al., 1982; Storrie et al., 1984a). With chases, HRP cytochemical deposits can be found in a second class of vesicles in which deposits are luminal (Storrie et al., 1984a). These luminal deposits identify a lysosomal compartment. The apparent membrane association of HRP cytochemical deposits in endocytic vesicles raises two possible explanations, 1) localization of cytochemical deposits close to the vesicle membrane is an artifact of the staining or fixation procedures or 2) HRP binds to sites on vesicle membrane.

This work was undertaken to determine if the HRP is membrane associated in endocytic vesicles in Chinese hamster ovary cells, a cell line which has no surface

receptors for HRP. We give evidence which indicates the presence of a reversible binding site in endocytic vesicles for HRP. This binding site(s) may also represent a molecule specific for this vesicle population.

Materials and Methods

Cell Culture

Chinese hamster ovary CHO-S(C2) cells were grown in suspension culture in αMEMFC10 (Eagle's Minimal Essential Medium, alpha modification without ribonucleosides and deoxyribonucleosides (αMEM) containing 10% heat inactivated fetal calf serum (FC10)) (Pool et al., 1983). Cell number was determined using a hemacytometer.

Periodate Treatment of HRP

HRP at a concentration of 20 mg/ml (type II, Sigma Chemical Co. St. Louis, MO) was incubated with 20 mM sodium metaperiodate in 0.1 M sodium acetate, pH 4.5 for 16-18 h at 4°C. The periodate reaction was stopped by the addition of ethylene glycol to 1.8 M followed by chromatography on a Sephadex G-25 M column (Pharmacia, PD-10) equilibrated with 75 mM sodium borate buffer, pH 8.0. The green colored, HRP rich fraction was collected, sodium borohydride was added, in borate buffer at a concentration of 0.6-1.0 mg/ml and the mix reacted at 4°C for 16-20 h to reduce aldehyde groups on carbohydrate residues. The reaction was stopped by chromatography on a Sephadex column, equilibrated with 0.9% sodium chloride. The green colored HRP rich fraction was collected and excess hydride

was eliminated by the addition of two drops of acetone. Mock treated HRP was processed similarily with the deletion of periodate and borohydride from the buffers. To test the efficacy of the periodate treatment, HRP subjected to periodate and borohydride was incubated with concanavalin A-agarose. The concanavalin A is specific for binding mannose residues on the HRP. After periodate treatment, HRP failed to bind to concanavalin A (Table 1).

HRP Uptake

Cells were incubated with HRP in serum supplemented media as previously described (Storrie et al., 1984a). Cell concentration was 0.5-3 x 10^7/ml and HRP concentration was 1mg/ml. Uptake was stopped by pouring cells onto two-fifths volume of crushed, frozen NKMC10 (NKM, 0.13M NaCl, 5mM KCL, 5mM $MgCl_2$, supplemented with 10% calf serum) and diluted 2-4 fold with chilled NKMC10. Cells were then washed 4 times with NKMC10 at 4^oC.

HRP Chase

Cells were resuspended in αMEMFC10 at either 37^oC or 17^oC. In some cases, the chase media contained drugs, as described in the figures. The 37^oC chases were stopped by pouring the culture onto crushed, frozen NKMC10. 17^oC chases were stopped by dilution with 4^oC NKM.

Table 1

Effect of Periodate Treatment on Horseradish Peroxidase Binding to Concanavalin A-agarose[1]

Treatment	µg HRP bound/ml of supernatant
None	6.27
Mock	5.50
Periodate	0.00

[1]HRP (10 µg/ml) was incubated for 15 minutes at room temperature in the absence or presence of concanavalin A-agarose resin in NKM with 0.9 mg/ml BSA. After 15 minutes, the agarose resin had setteled and the supernatant was collected. The micrograms of HRP in the supernatant was determined based on the activity of known amounts of "appropriately" treated HRP. The presence of α-methylmannoside (13 mM) completely blocked binding to concanavalin A.

Postnuclear Supernatant Preparation(PNS)

Cells were washed once with NKM and once with 0.25 M sucrose at 4°C. The cell suspension, in 0.25 M sucrose was then placed in a N_2 cavitation apparatus and a postnuclear supernatant (PNS) was prepared as previously described (Pool et al., 1983).

Hypotonic Lysis Assay For Membrane Association

For hypotonic lysis, the PNS prepared in 0.25 M sucrose was brought to a final sucrose concentration of 0.05 M by the addition of either deionized water, 5 mM sodium phosphate buffer (pH 6.7) or 5 mM sodium acetate buffer (pH 4.6). In the case of detergent or carbonate lysis, zwitterionic detergent (Zwittergent detergent 3-12, Calbiochem, San Diego, CA) or sodium carbonate (pH 11.3), (Storrie et al., 1984b) was added to the PNS to a final concentration of 1% or 100 mM respectively. The diluted PNS was then incubated 15 minutes at the indicated temperature and then pelleted at 111,000 x g_{max} for 1 h in a Ti50 rotor (Beckman).

HRP Cytochemistry

HRP pulse labeled cells were processed for electron microscopic cytochemistry using the reaction with diaminobenzidine (DAB) as described previously (Storrie et

al., 1984a).

Enzyme Assays

HRP activity was assayed using an o-dianisidine substrate in the presence of 0.1% Triton X-100 (Pool et al., 1983). β-hexosaminidase (Pool et al., 1983) and β-galactosidase (Rome et al., 1979) were assayed using a 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside and a 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-galactopyranoside fluorescent substrates respectively also in the presence of 0.1% Triton X-100.

HRP Exocytosis

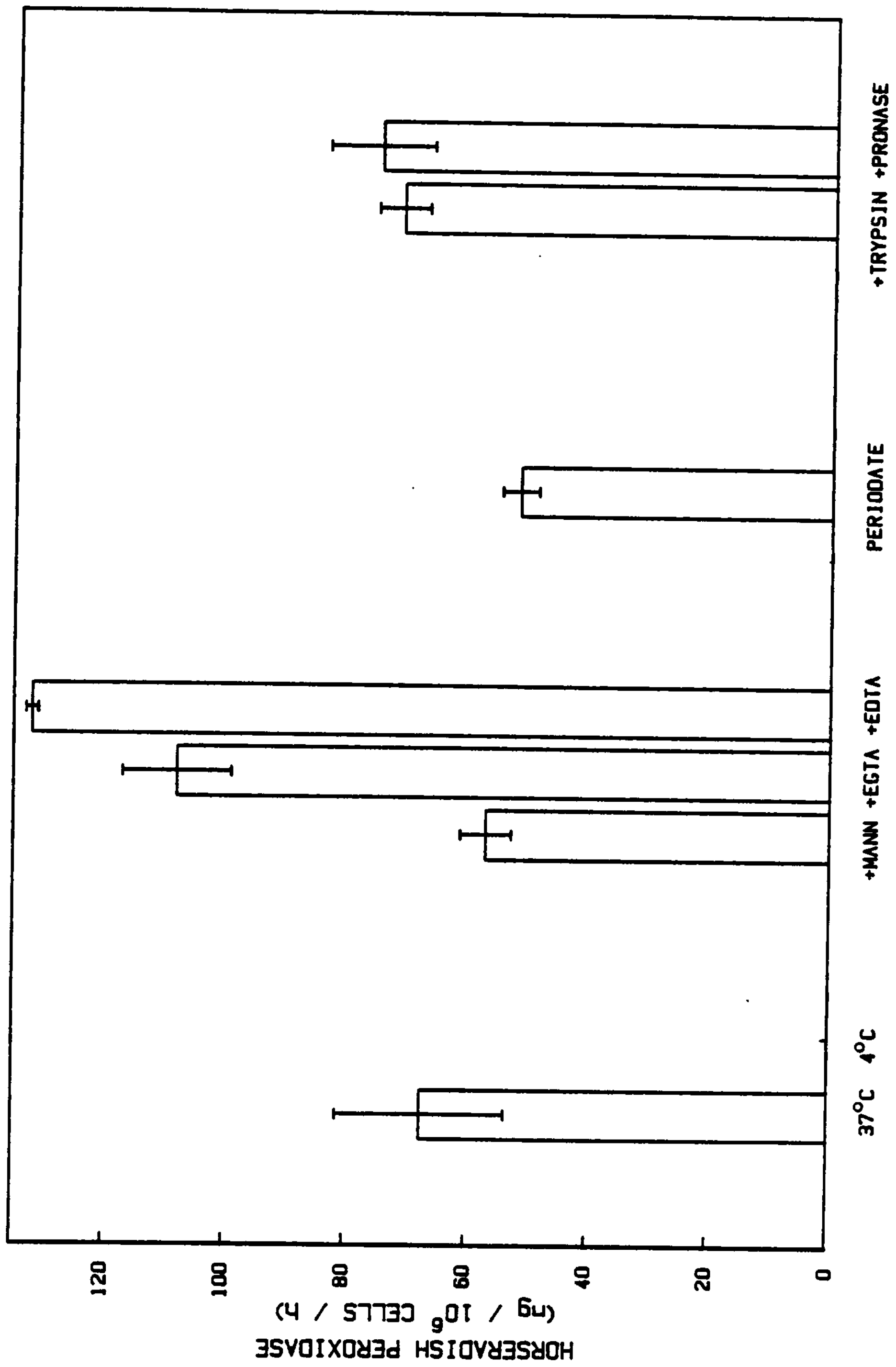
Pulsed cells with or without chelators were resuspended in saline supplemented with glucose and BSA for 30 minutes and the extent of exocytosis determined (Adams et al., 1982).

Results

Mode of HRP Internalization

Internalization is a temperature dependent process (Adams et al., 1982; for review see Pastan and Willingham, 1985). As shown in Figure 1, HRP uptake by CHO cells was significantly decreased by reducing temperature from 37°C to 4°C. To determine if HRP must bind to plasma membrane prior to internalization in this cell system, we attempted to inhibit the uptake of HRP by treatments which should effect uptake through a mannose/N-acetylglucosamine or other receptors. HRP uptake by mannose/N-acetylglucosamine receptors in other cell types is inhibited by high concentrations of yeast mannan (Achord et al., 1977) and is dependent on Ca^{2+} (Townsend and Stahl, 1981). At a high concentration of yeast mannan (19 mg/ml) no effect on uptake of HRP occurred (Figure 1). In the presence of EGTA Mg or EDTA an actual increase of 60%-96% in overall uptake occurred (Figure 1). Investigating the cause for the high overall uptake values, we found a 10-15% decrease in exocytosis in the presence of these chelators (data not shown). It has been suggested that a small decrease in exocytosis can result in significant increases in overall uptake values (Swanson et al., 1985). From these data we conclude that little, if any, HRP uptake is mediated by a

Figure 1. Uptake of HRP Under Conditions Which Should Block Mannose/N-acetylglucosamine Receptor Binding. CHO cells were incubated for one hour with 1 mg/ml HRP or 0.45 mg/ml periodate treated HRP at 37°C. Mannan was present during the uptake at 19 mg/ml. EGTA Mg and EDTA (disodium salt) were present at 5 mM. For trypsin and pronase treatments, cells were incubated prior to uptake with 20 µg/ml trypsin or pronase respectively. Following uptake, cells were lysed using 0.05% Triton X-100 and HRP was assayed as described in Materials and Methods. The average 37°C uptake value is an average of 8 separate determinations. All other values are the average of duplicates. To compare uptakes from different experiments, values were normalized on the basis of the 37°C control value. Bars represent the range of uptake values.



HORSERADISH PEROXIDASE
(ng / 10^6 CELLS / h)
0
20
40
60
80
100
120
37°C
4°C
+MANN
+EGTA
+EDTA
PERIODATE
+TRYPSIN
+PRONASE

mannose/N-acetylglucosamine specific process.

As a final test for possible involvment of a sugar specific receptor in HRP uptake in CHO cells, the uptake of native HRP and periodate treated HRP were compared. Periodate treatment destroys the ring structure of saccharides which have hydroxyl groups on adjacent ring carbons. Periodate treatment depressed uptake slightly (~20%). In general, receptors are protein molecules and therefore may be protease sensitive. To test if a protease sensitive molecule mediates HRP internalization, CHO cells were pretreated with trypsin or pronase before incubation with HRP. No differences in uptake from control cells was seen (Figure 1). All of these data support previous evidence that HRP uptake is by fluid phase endocytosis in these cells.

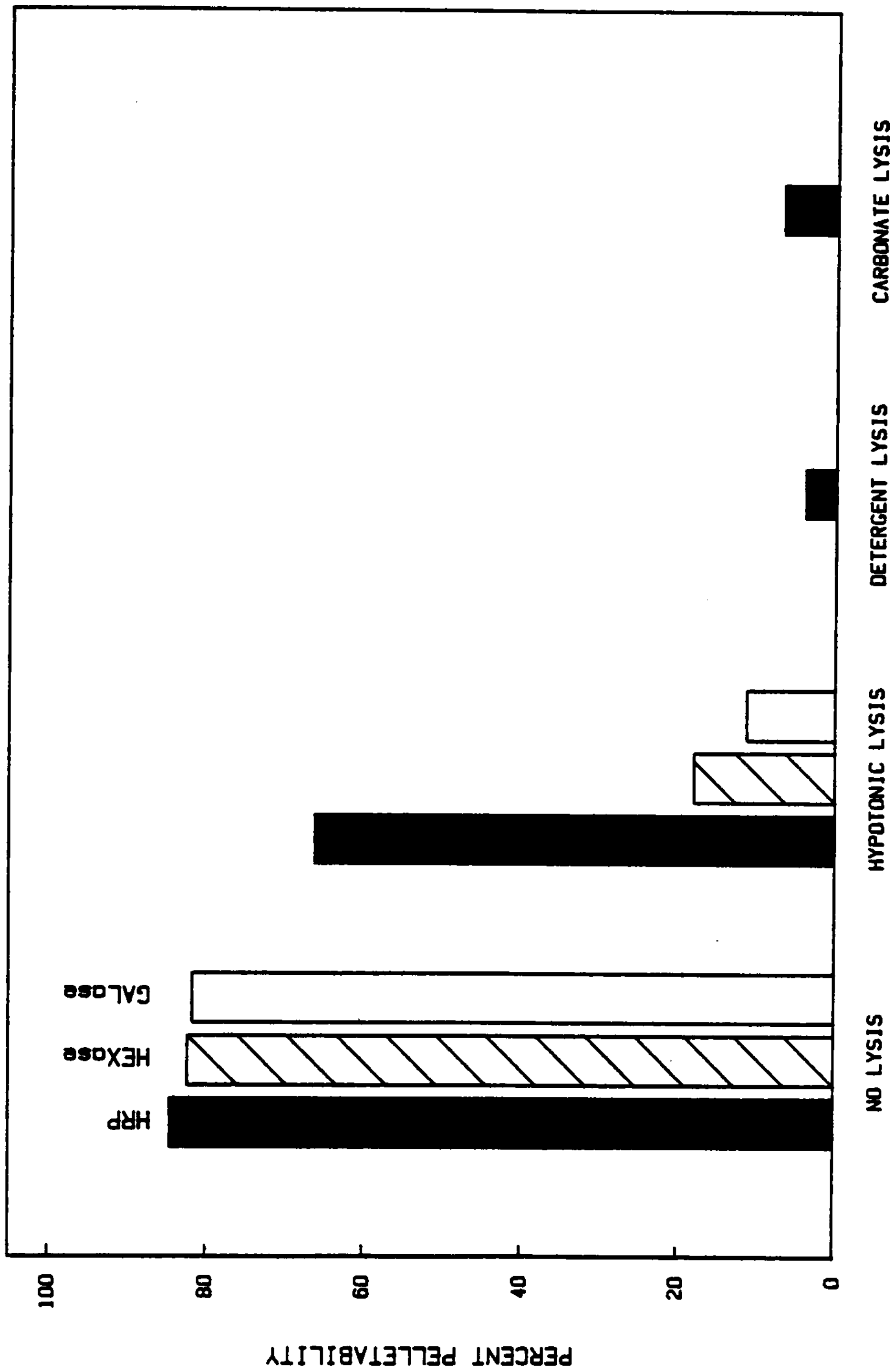
Demonstration of HRP Binding to Endocytic Vesicle Membrane

The diaminobenzidine deposits arising from HRP cytochemistry are associated with endocytic vesicle membrane in fibroblasts (Steinman et al., 1974; Storrie et al., 1984a). As a first test of whether this association reflects an actual membrane association of the enzyme, we used a hypotonic lysis assay to determine whether the HRP pelleted with membranes. Total postnuclear supernatants in 0.25 M sucrose were diluted to 0.05 M sucrose

(hypotonic) with deionized water at 4°C and the lysed preparations were then centrifuged. Figure 2 demonstrates the extent of enzyme pelletability (i.e. a putative measure of membrane association) of HRP (10 minute pulse). For the initial postnuclear supernatant, HRP and lysosomal enzymes (β-hexosaminidase and β-galactosidase) were greater than 80% pelletable. Enzyme activity found in the supernatant (i.e. soluble) was most likely from disrupted vesicles and lysosomes. Upon lysing at neutral pH (Figure 2), approximately two-thirds of the HRP remained associated with vesicle membrane while 80-90% of lysosomal enzymes were found in the supernatant. A portion of the HRP found in the supernatant may be associated with membrane fragments too small to readily sediment. Under similar conditions, only 85%-90% of alkaline phosphodiesterase I, an enzyme known to be a membrane protein, can be pelleted (Sachdeva and Storrie, unpublished observation).

To further test whether HRP pelleting is due to membrane association, the effect of dissolving organelle membrane with a zwitterionic detergent, which does not disrupt protein-protein interaction (Hoogeveen et al., 1983), was determined. Less than 10% of the HRP was pelleted following disruption of the membrane with detergent (Figure 2). This suggests that HRP

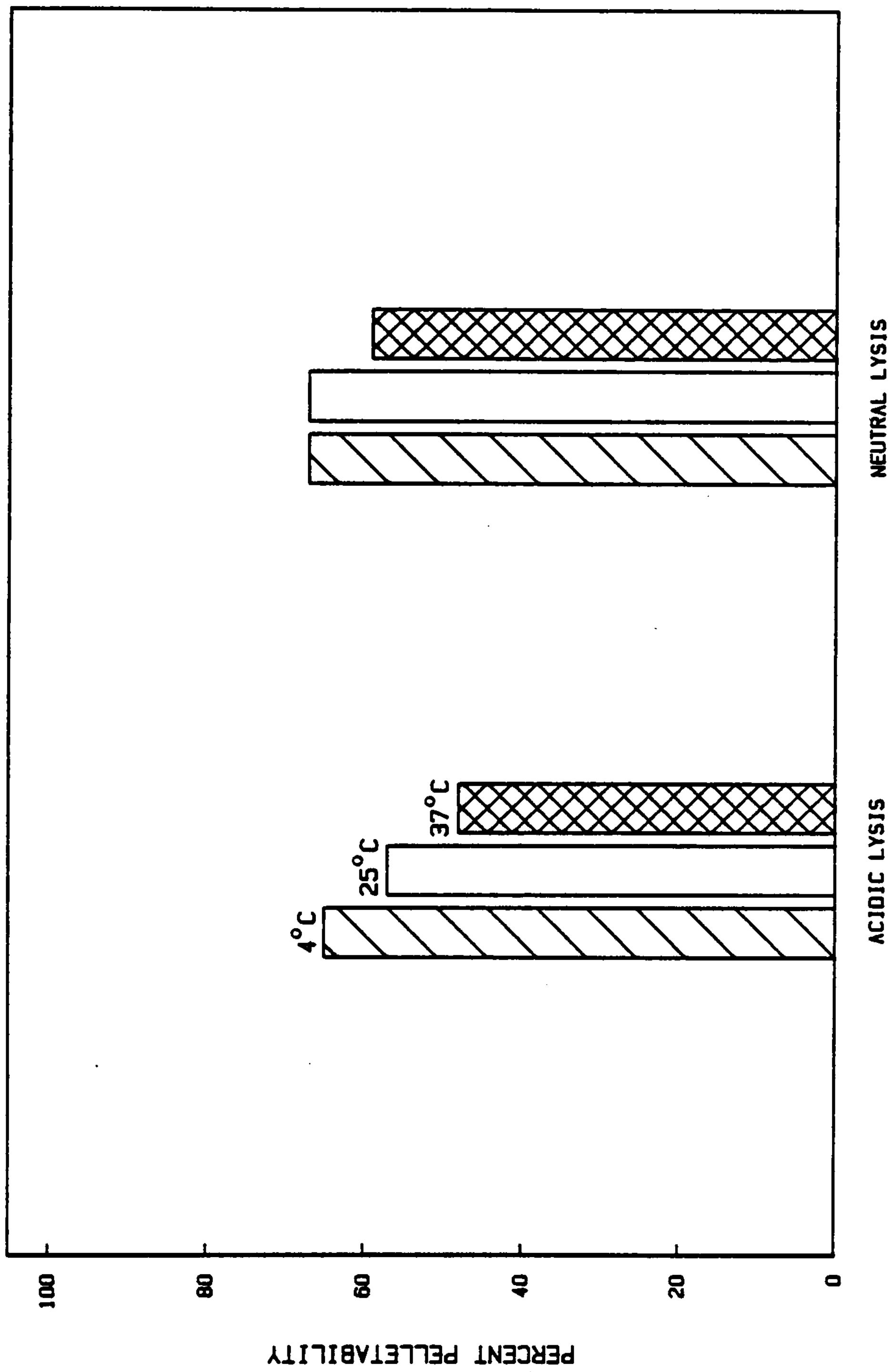
Figure 2. Enzyme Pelletability Before and After Lysis. CHO cells were pulsed with HRP for 10 minutes at 37°C. Postnuclear supernatants were prepared and lysed at 4°C, by dilution with deionized water to 0.05 M sucrose, 1% zwitterionic detergent or 100 mM sodium carbonate. In the controls (no lysis), postnuclear supernatants were kept in 0.25 M sucrose (isotonic). Controls and lysed preparations were then pelleted and the activity of horseradish peroxidase (HRP), β-hexosaminidase (HEXase) and β-galactosidase (GALase) was determined.


HRP
HEXase
GALase
PERCENT PELLETABILITY
100
80
60
40
20
0
NO LYSIS
HYPOTONIC LYSIS
DETERGENT LYSIS
CARBONATE LYSIS

pelletability is due to association with the membrane of early endocytic vesicles. Lysis of vesicles (by high pH) using a carbonate solution results in stripping membranes of peripheral proteins (Fujiki et al., 1982; Howell and Palade, 1982). The low pelletability of HRP (<10%) seen with carbonate treatment suggests that HRP may be associated with membranes through weak electrostatic interactions rather than covalent bonds or hydrophobic interactions (Figure 2).

H^+ ion concentration has a significant effect on reversible binding in receptor systems. Physiological dissociation of ligands from receptor at pH<6.0 has been demonstrated (Hudgin et al., 1974; Posner et al., 1977; Gonzalez-Noriega et al., 1980; Maxfield, 1982). Therefore endocytic vesicles from cells pulsed with HRP for 10 minutes were hypotonically lysed under different conditions of pH and temperature (Figure 3). The data in Figure 3 demonstrate that at pH 4.6, pelletability of HRP was slightly lower at all temperatures tested. At acidic pH, as temperature increased from 4°C to 37°C, HRP pelletability decreased. No consistant variation in pelletability was seen at neutral pH with a change in temperature. Together these results suggest that HRP should dissociate slightly faster from endocytic vesicle membrane at acidic pH than at neutral pH at physiological

Figure 3. The Effect of pH and Temperature on HRP Pelletability in a Vesicle Preparation. Cells were pulsed with HRP for 10 minutes at 37°C. Postnuclear supernatants were prepared and lysed at acidic pH with 5 mM sodium acetate buffer (pH 4.6) or at neutral pH with either deionized water or 5 mM sodium phosphate buffer (pH 6.7) at 4°C, room temperature ($\sim$25°C) or 37°C. The lysed preparations were pelleted and the percentage of HRP in the membrane pellet was determined.


4°C
25°C
37°C
ACIDIC LYSIS
NEUTRAL LYSIS
PERCENT PELLETABILITY
0
20
40
60
80
100

temperatures.

Is HRP Bound to The Mannose/N-acetylglucosamine Receptor?

To test whether HRP in endocytic vesicles may be associated with a mannose/N-acetylglucosamine receptor or any other sugar specific receptor, vesicles were prepared from cells which had internalized either native HRP in the presence of 19 mg/ml yeast mannan or periodate treated HRP (Figure 4). Periodate treatment should prevent binding to any sugar specific receptor. In both cases, a high level of HRP membrane association was observed in the hypotonic lysis assay. This result is consistant with electron microscopic cytochemistry in which HRP cytochemical deposits in endocytic vesicles were classified as either rimmed, i.e. deposits very close to vesicle membrane, or luminal (Figure 5). For cells pulsed with native HRP for 10 minute in the absence or presence of excess yeast mannan or pulsed with periodate treated HRP, vesicles having deposits which rim the luminal surface accounted for 70-80% of the vesicles scored (Table 2), under all conditions.

By comparing cytochemical staining patterns for HRP, it has been observed that deposits rim endocytic vesicles while luminal deposits are observed in lysosomes (Steinman et al., 1976; Storrie et al., 1984a). Apparently HRP

Figure 4. Effect of Yeast Mannan and Periodate Treatment of HRP on Pelletability of Enzyme in Endocytic Vesicles. Cells were incubated with either HRP (1 mg/ml) or periodate treated HRP (0.5 mg/ml) for 10 minutes at 37°C. In the case of +mannan incubation, yeast mannan (19 mg/ml) was present during the uptake. Postnuclear supernatants were prepared and lysed hypotonically with 5 mM sodium phosphate buffer (pH 6.7) at room temperature and the percentage of HRP associated with the membrane pellet was determined. The result of lysis at pH 6.7 using room temperature buffer (5 mM) is included from figure 3 as a reference.

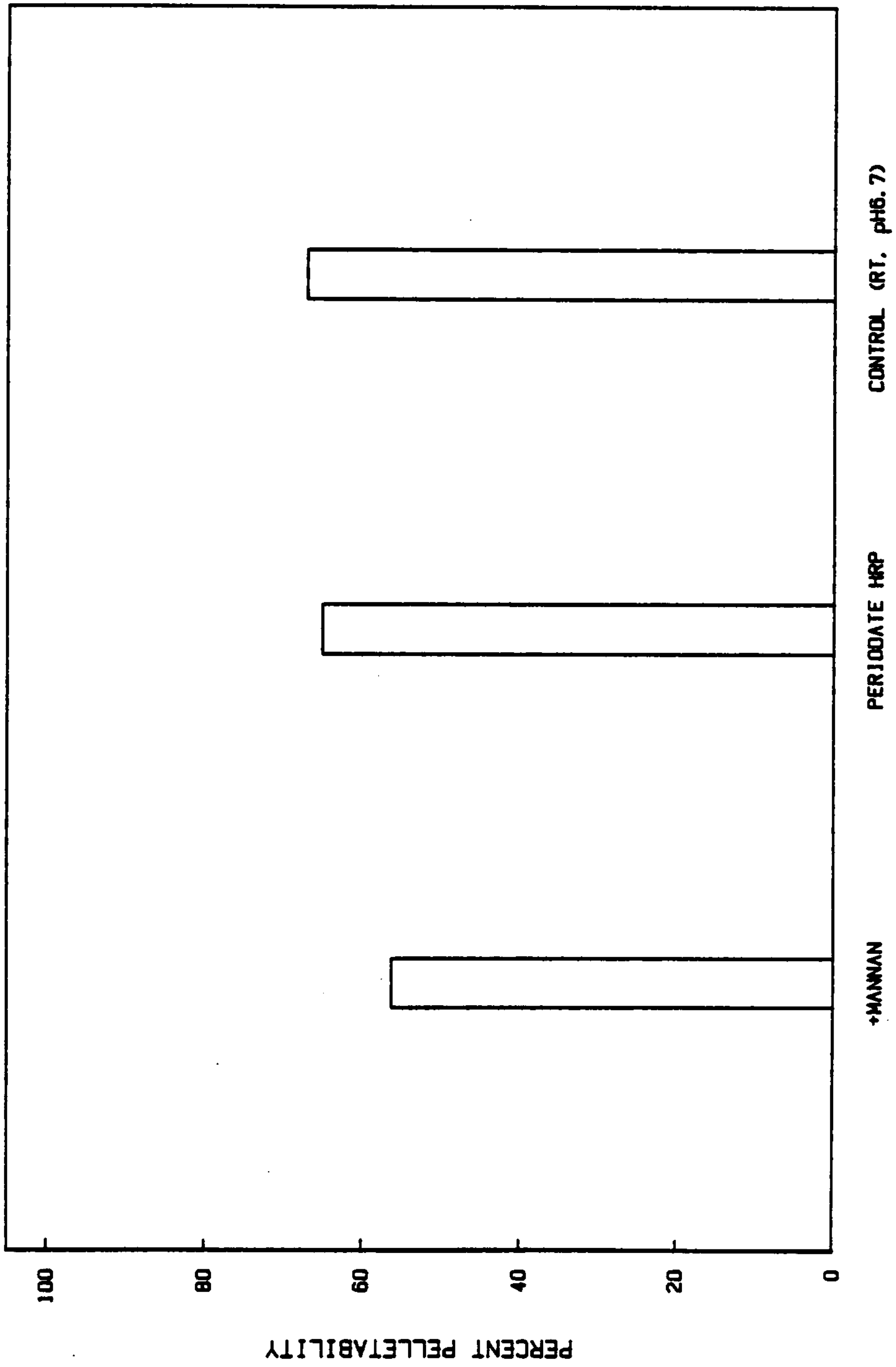

PERCENT PELLETABILITY
0
20
40
60
80
100
+MANNAN
PERIODATE HRP
CONTROL (RT. pH6.7)

Figure 5. Cytochemical Localization of HRP in CHO Cells. CHO cells were pulsed for 10 minutes with HRP in the presence of 19 mg/ml yeast mannan. After the pulse, the cells were washed with NKMC10 and NKM and then processed for HRP cytochemistry. The black deposits represent sites of HRP activity. Vesicles which have deposits which rim their luminal surface are labeled 'R' and those with deposits located in the lumen are labeled 'L'. Bar, 0.5 μM. x50,500.

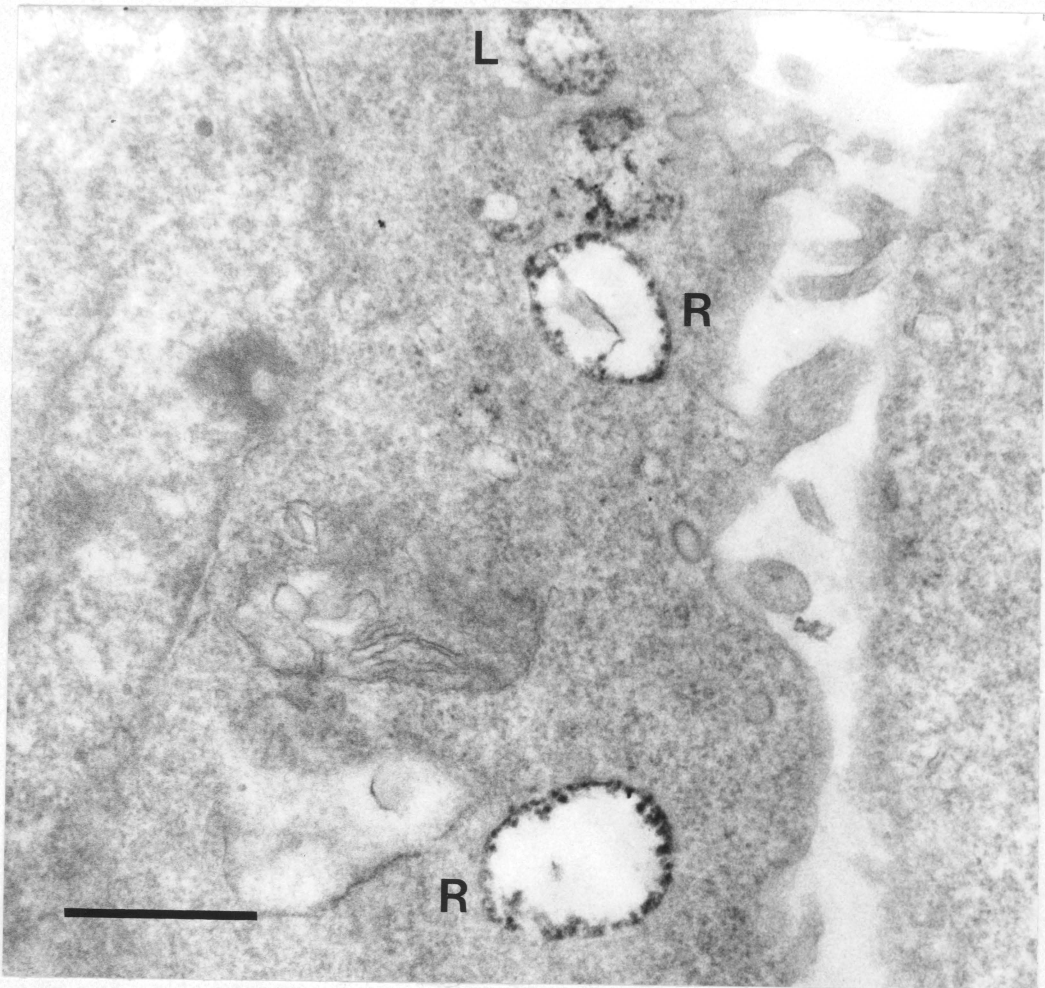

L
R
R

Table 2

Effect of Mannan and Periodate Treatment on Apparent Membrane Association of Horseradish Peroxidase Cytochemical Deposits in Endocytic Vesicles[1]

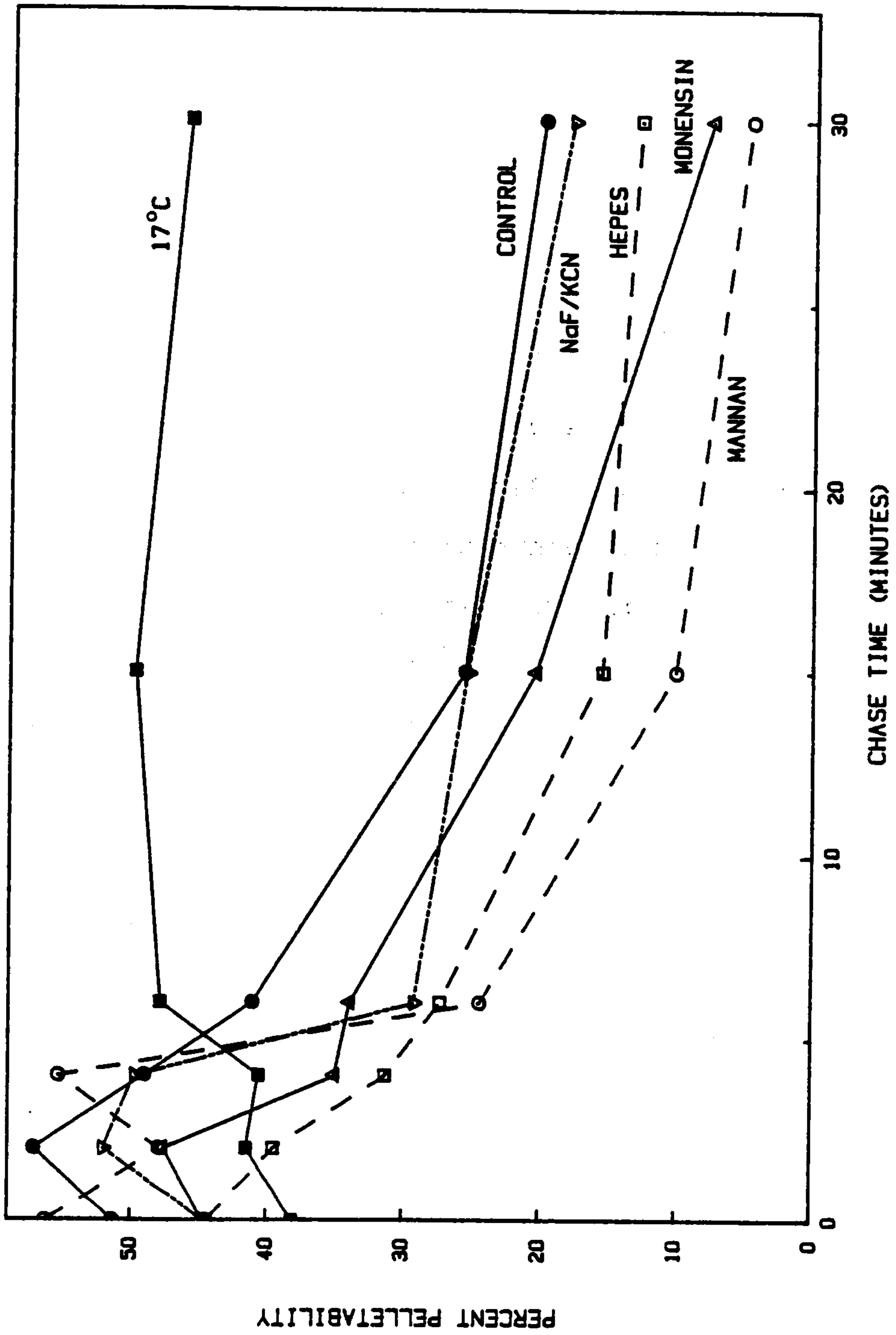
Pulse Conditions	Deposits (%) luminal	Deposits (%) rimmed	Vesicles Scored Total No.
37°C	23	77	83
+Mannan	20	80	83
Periodate Treated HRP	17	73	12

[1]Native or periodate treated HRP (1 mg/ml) was incubated with cells at 37°C for 10 minutes. Uptakes were in α FC10 or 0.9% NaCl respectively, in the absence or presence of yeast mannan (19 mg/ml). Uptake was stopped and cells were processed for HRP cytochemistry. Vesicles observed under electron microscopy were scored as either having deposits which rimmed the luminal surface or deposits localized luminaly.

dissociates from vesicle membrane at some point during the transport process. The degree of membrane dissociation of HRP, internalized during a 10 minute pulse over a chase sequence was investigated (Figure 6). Only small decreases in pelletability of HRP were observed in the first 2-4 minutes. Over 80% of the total decrease in pelletability occurred between 2-15 minutes of chase. From 15-30 minutes, pelletability decreased slightly while from 30-45 minutes (data not shown) no change was seen. By 15 minutes, it can be shown by cell fractionation that HRP is in lysosomes (Pool et al., 1983; see chapter III). It has also been demonstrated in our laboratory that after lysis, the pelletability of HRP present in lysosomes is less than 10% (Buckmaster and Storrie, unpublished observation).

The use of putative elevators of vesicle pH, HEPES and monensin, as well as ATP production inhibitors NaF/KCN were ineffective in significantly inhibiting HRP dissociation (Figure 6). HRP uptake in the presence of yeast mannan also did not result in an inhibition of the dissociation of HRP from vesicle membrane. Reducing temperature to 17°C during the chase prevented HRP dissociation from vesicle membrane during a 30 minute chase.

Figure 6. <u>Kinetics of Dissociation of HRP From Vesicle Membrane.</u> Cells were incubated with HRP for 10 minutes at 37°C. For HEPES (40 mM) and mannan (19 mg/ml) treatment, both were present during the pulse period. Uptake was stopped and cells were washed in the cold. Cells were then warmed to 37°C or 17°C in MEMFC10 (chase). Monensin was at a concentration of 10 uM and NaF/KCN were at concentrations of 2 mM and 1 mM respectively during the chase period. For the HEPES treated cells, HEPES (40 mM) was also present in the chase medium. The postnuclear supernatant from cells at each chase time was hypotonically lysed using 5 mM phosphate buffer (pH 6.7) at room temperature. Membranes were pelleted and the percent of HRP determined. Control (●———●), 17°C chase (■———■), HEPES (□– – –□), monensin (△———△), NaF/KCN (▽- - - -▽), mannan (○– – –○).


17°C
CONTROL
NaF/KCN
HEPES
MONENSIN
MANNAN
CHASE TIME (MINUTES)
0
10
20
30
PERCENT PELLETABILITY
0
10
20
30
40
50

Discussion

Internalized horseradish peroxidase (HRP) generates membrane associated cytochemical deposits in endocytic vesicles. The location of these deposits may represent an artifact of the cytochemical procedure or a true membrane association of enzyme. The evidence presented here indicates that although HRP is internalized through fluid phase endocytosis, it becomes associated with vesicle membrane upon entering an endocytic vesicle compartment. HRP then dissociates from vesicle membrane in a pH independent but temperature sensitive process, becoming soluble again in lysosomes. Overall this evidence suggests specific compartmentalization of HRP binding site(s) to endocytic vesicles.

Previous work indicates HRP is internalized by fibroblasts through fluid phase endocytosis (Steinman et al., 1974; Adams et al., 1982; Storrie et al., 1984a). Using a high concentration (19 mg/ml) of yeast mannan, divalent cation chelators or periodate treatment of the HRP to destroy the ring structure of sugar side chains, we find no evidence of receptor-mediated uptake of HRP by mannose/N-acetylglucosamine or any other sugar specific receptor. In the presence of divalent cation chelators (EGTA Mg or EDTA) an actual increase in HRP accumulation was observed. This may be due to decreased exocytosis;

exocytosis of HRP is decreased in the absence of calcium ions. We conclude that HRP internalization by CHO cells is by fluid phase endocytosis.

Once internalized through fluid phase endocytosis, HRP becomes membrane associated. Membrane association has been demonstrated by a hypotonic lysis assay. After hypotonic lysis of a postnuclear supernatant, internalized HRP is almost as pelletable as the known plasma membrane protein alkaline phosphodiesterase I. The HRP membrane association appears to be through weak electrostatic interactions with the vesicle membrane; detergent treatment to dissolve membrane solublizes HRP as does carbonate treatment used to strip peripheral proteins from membranes. The major isoenzyme of HRP has been shown to have a slight positive charge at neutral pH (Welinder, 1979) consistant with HRP binding by electrostatic interaction. Using yeast mannan at high concentration or periodate treated HRP, only a small effect is seen on membrane association (% pelletability) of HRP, suggesting HRP binding is not to the mannose/N-acetylglucosamine or other sugar specific receptor. By electron microscopic cytochemistry, HRP is membrane associated in the presence of yeast mannan or after periodate treatment. Under the conditions of the hypotonic lysis assay, the membrane association is temperature stable at neutral pH and at

pH 4.6 decreases slightly as lysis temperature is increased from 4°C to 37°C.

The gradual dissociation of HRP during a chase sequence occurs during a transport process in which HRP is first in an endocytic vesicle compartment and later in lysosomes. HRP in a lysosomal compartment is not membrane associated (Steinman et al., 1976; Pool et al., 1983; Buckmaster, Lo Braico and Storrie manuscript in preparation). This compartmentalization of HRP membrane association indicates that the molecule which constitutes an HRP binding site(s) is restricted to endocytic vesicles. As such, this binding site may constitute a molecular marker for endocytic vesicles. The actual chemical nature of the HRP binding site(s) in endocytic vesicles is presently unknown.

Dissociation of HRP from membrane is not inhibited by yeast mannan, pH elevators and NaF/KCN but is inhibited at 17°C. The lack of effect of pH elevators and NaF/KCN indicates that HRP dissociation from its binding site is a pH independent process. pH elevators and NaF/KCN effect HRP transport from endocytic vesicles to lysosomes (see Chapter III). Reduced temperature may be acting by either a direct effect on HRP dissociation or by inhibiting transport from one vesicle compartment to another. Slowing of transport to lysosomes at reduced temperature

has already been demonstrated in hepatocytes (Dunn et al., 1980) and is also true for CHO cells (see chapter III).

Swanson et al. (1985) have reported that HRP binding is capable of directing the flow of internalized fluid to a vesicle compartment, presumed to be lysosomes. This increased accumulation of fluid was shown to be independent of HRP binding to a mannose/N-acetylglucosamine receptor. Buckmaster, Lo Braico and Storrie (manuscript in preparation) have also shown that HRP stimulates solute uptake in CHO cells. It is possible that the HRP effect reported by these investigators is due to enzyme binding to endocytic vesicle membrane as reported here. This suggests that the HRP binding site(s) may have a physiologically significant role in directing endocytic membrane traffic.

References

Achord, D.T., Brot, F.E., Sly, W.S. (1977) Inhibition of the rat clearance system for agalacto-orosomucoid by yeast mannans and by mannose. Biochem. Biophys. Res. Commun., 77:409-415.

Adams, C.J., Maurey, K.M., Storrie, B. (1982) Exocytosis of pinocytic contents by Chinese hamster ovary cells. J. Cell Biol., 93:632-637.

Baynes, J.W. and Wold, F. (1976) Effect of glycosylation on the in vivo circulating half-life of ribonuclease. J. Biol. Chem., 251:6016-6024.

Clarke, J. and Shannon, L.M. (1976) The isolation and characterization of the glycopeptides from horseradish peroxidase isoenzyme. Biochim. Biophys. Acta, 427:428-442.

Dunn, W.A., Hubbard, A.L., Aronson, N.N.Jr. (1980) Low temperature selectively inhibits fusion between pinocytic vesicles and lysosomes during heterophagy of ^{125}I-asialofetuin by the perfused rat liver. J. Biol. Chem., 255:5971-5978.

Fujiki, Y., Hubbard, A.L., Fowler, S., Lazarow, P.B. (1982) Isolation of intracellular membranes by means of sodium carbonate treatment: Application to endoplasmic reticulum. J. Cell Biol., 93:97-102.

Gonzalez-Noriega, A., Grubb, J.H., Talkad, V., Sly, W.S. (1980) Chloroquine inhibits lysosomal enzyme pinocytosis and enhances lysosomal enzyme secretion by impairing receptor recycling. J. Cell Biol., 85:839-852.

Hoogeveen, A.T., Verheijen, F.W., Galjaard, H. (1983) The relation between human lysosomal β-galactosidase and its protective protein. J. Biol. Chem., 258:12143-12146.

Howell, K.E. and Palade, G.E. (1982) Hepatic golgi fractions resolved into membrane and content subfractions. J. Cell Biol., 92:822-832.

Hudgin, R.L., Pricer, W.E.Jr., Ashwell, G., Stockert, R.J., Morell, A.G. (1974) The isolation and properties of a rabbit liver binding protein specific for asialoglycoproteins. J. Biol. Chem., 249:5536-5543.

Kaplan, J. and Nielsen, M. (1978) Pinocytic activity of

rabbit alveolar macrophages in vitro. J. Reticuloendothel. Soc., 24:673-685.

Lang, T. and de Chastellier, C. (1985) Fluid phase and mannose receptor-mediated uptake of horseradish peroxidase in mouse bone marrow-derived macrophages. Biochemical and ultrastructural study. J. Cell Biol., 53:149-154.

Maxfield, F.R. (1982) Weak bases and ionophores rapidly and reversibly raise the pH of endocytic vesicles in cultured mouse fibroblasts. J. Cell Biol., 95:676-681.

Pastan, I. and Willingham, M.C. (1985) The pathway of endocytosis. In: Endocytosis. I. Pastan and M.C. Willingham eds. Plenum Press, New York. pp. 1-44.

Pool, R.R., Maurey, K.M., Storrie, B. (1983) Characterization of pinocytic vesicles from CHO cells: Resolution of pinosomes from lysosomes by analytical centrifugation. Cell Biol. Int. Rep., 7:361-367.

Posner, B.I., Josefsbert, Z., Bergeron, J.J.M. (1977) Characterization of insulin binding sites in golgi fractions from the liver of female rats. J. Biol. Chem., 253:4067-4073.

Rodman, J.S., Schlesinger, P., Stahl, P. (1978) Rat plasma clearance of horseradish peroxidase and yeast invertase is mediated by specific recognition. FEBS Lett., 85:345-348.

Rome, L.H., Garvin, A.J. Allietta, M.M., Neufeld, E.F. (1979) Two species of lysosomal organelles in cultured human fibroblasts. Cell, 17:143-153.

Steinman, R.M., Brodie, S.E., Cohn, Z.A. (1976) Membrane flow during pinocytosis. Stereologic analysis. J. Cell Biol., 68:665-678.

Steinman, R.M. and Cohn, Z.A. (1972) The interaction of soluble horseradish peroxidase with mouse peritoneal macrophages in vitro. J. Cell Biol., 55:186-204.

Steinman, R.M., Silver, J.M., Cohn, Z.A. (1974) Pinocytosis in fibroblasts. Quantitative studies in vitro. J. Cell Biol., 63:949-969.

Stockert, R.J., Morell, A.G., Scheinberg, I.H. (1976) The existance of a second route for the transfer of certain

glycoproteins from the circulation into the liver. Biochem. Biophys. Res. Commun., 68:988-993.

Storrie, B., Pool, R.R.Jr., Sachdeva, M., Maurey, K.M., Oliver, C. (1984a) Evidence for both prelysosomal and lysosomal intermediates in endocytic pathways. J. Cell Biol., 98:108-115.

Storrie, B., Sachdeva, M., Viers, V.S. (1984b) Chinese hamster ovary cell lysosomes retain pinocytized horseradish peroxidase and in situ-radioiodinated proteins. Mol. Cell. Biol., 4:296-301.

Sung, S.- S.J., Nelson, R.S., Silverstein, S.C. (1983) The role of the mannose/N-acetylglucosamine receptor in the pinoytosis of horseradish peroxidase by mouse peritoneal macrophages. J. Cell. Physiol., 116:21-25.

Swanson, J.A., Yirinec, B.D., Silverstein, S.C. (1985) Phorbol esters and horseradish peroxidase stimulate pinocytosis and redirect the flow of pinocytosed fluid in macrophages. J. Cell Biol., 100:851-859.

Townsend, R. and Stahl, P. (1981) Isolation and characterization of a mannose/N-acetylglucosamine/fucose-binding protein from rat liver. Biochem. J. 19:209-214.

Welinder, K.G. (1979) Amino acid sequence studies of horseradish peroxidase. Amino and carboxyl termini, cyanogen bromide and tryptic fragments, the complete sequence, and some structural characteristics of horseradish peroxidase. Eur. J. Biochem., 96:483-502.

Chapter III

Effects of Temperature, pH Elevators and Energy Production Inhibitors Upon Horseradish Peroxidase Transport Through Endocytic Vesicles.

Introduction

In both fluid phase and receptor-mediated systems endocytic vesicles become acidic (Tycko and Maxfield, 1982; Galloway et al., 1983; Merion et al., 1983; Murphy et al., 1984). In receptor-mediated endocytosis, substances which raise vesicular pH are capable of disrupting normal transport to lysosomes as well as receptor reutilization (Gonzalez-Noriega et al., 1980; Anderson et al., 1982; Harford et al., 1983; Merion and Sly, 1983; Schwartz et al., 1984). Evidence suggests that disruption of normal processing events by pH elevators may be due to inhibition of ligand-receptor dissociation in an endocytic compartment (Tycko and Maxfield, 1982). Dunn et al. (1980) have demonstrated in an asialoglyoprotein receptor system a potential temperature sensitive step(s). Fusion of endocytic vesicles and lysosomes was inhibited below 20°C. Wolkoff et al. (1984) have shown using reduced temperature, monensin, Na^+ deprivation and colchicine/cytochalasin B that transport of asialoglycoproteins to lysosomes can be separated into a number of

different steps.

In the previous chapter, horseradish peroxidase (HRP) has been shown to enter Chinese hamster ovary (CHO) cells, a cell line of fibroblastic origin, by fluid phase endocytosis. However, once internalized, HRP is rapidly transfered into endocytic vesicles which appear to have HRP binding sites. HRP binds to vesicle membrane and then, as transport occurs, gradually dissociates in a pH independent but temperature dependent manner from the membrane. HRP in binding specifically to an endocytic vesicle compartment represents the first example of a novel event in endocytic transport.

The route of transport for HRP from plasma membrane through endocytic vesicle(s) to lysosomes is well established (Steinman et al., 1976; Storrie et al., 1984a). However, less is known about the mechanism(s) and total number of individual steps involved. In this study we have used reduced temperature, the pH elevators NH_4Cl, monensin, and HEPES as well as the metabolic poisons NaF/KCN in an effort to 1) determine the effect of temperature, pH and ATP depletion on transport of HRP, 2) attempt to locate individual steps which are sensitive to particular inhibitors and 3) to define a minimum number of transport steps present in this system.

Materials and Methods

Cell Culture

Suspension cultured Chinese hamster ovary cells were grown in αMEMFC10 as described in the previous chapter.

HRP Pulse and Chase

Pulse: Cells at a concentration between 7.5 X 10^5-4.5 X 10^7 cells/ml in αFC10 were incubated with HRP (0.5 or 1 mg/ml) for 3 or 10 minutes at 37°C. For uptake in the presence of HEPES (N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid), the HEPES concentration was 20 mM or 40 mM. Uptake was stopped by pouring cells onto two-fifths volume of crushed frozen saline supplemented with 10% calf serum (NKMC10, see preceeding chapter). The cells were then diluted 3.5 fold with 4°C saline (NKM) and washed with cold saline.

Chase: Cells were resuspended in αMEM with or without 10% fetal calf serum. Chase periods, in the absence or presence of putative transport inhibitors, were for 3-45 minutes at 17°C or at 37°C. The chases were stopped by pouring cells onto crushed frozen saline as above. For experiments using monensin, a stock was prepared at a concentration of 1.4 mM monensin in absolute ethanol.

Continuous Uptake

7.5 X 10^5 cells/ml suspended in α MEMFC10 (17°C or 37°C), with 100 I.U./ml penicillin and 100 μg/ml streptomycin (Flow Laboratories, Inc. McLean, Virginia 22102) were exposed to 0.5 mg/ml HRP for 6 or 18 hours. For uptake in the presence of HEPES, the HEPES concentration was 20 mM. Uptake was stopped by pouring cells onto one-fifth or two-fifth volume crushed frozen NKMC10 and the cells further diluted 1.4-1.5 fold with 4°C NKM.

Cell Fractionation

Washed cells were disrupted by N_2 cavitation and a postnuclear supernatant prepared as described in the previous chapter. The postnuclear supernatant was then fractionated on 10% Percoll density gradients (Pool et al., 1983).

Enzyme Assays

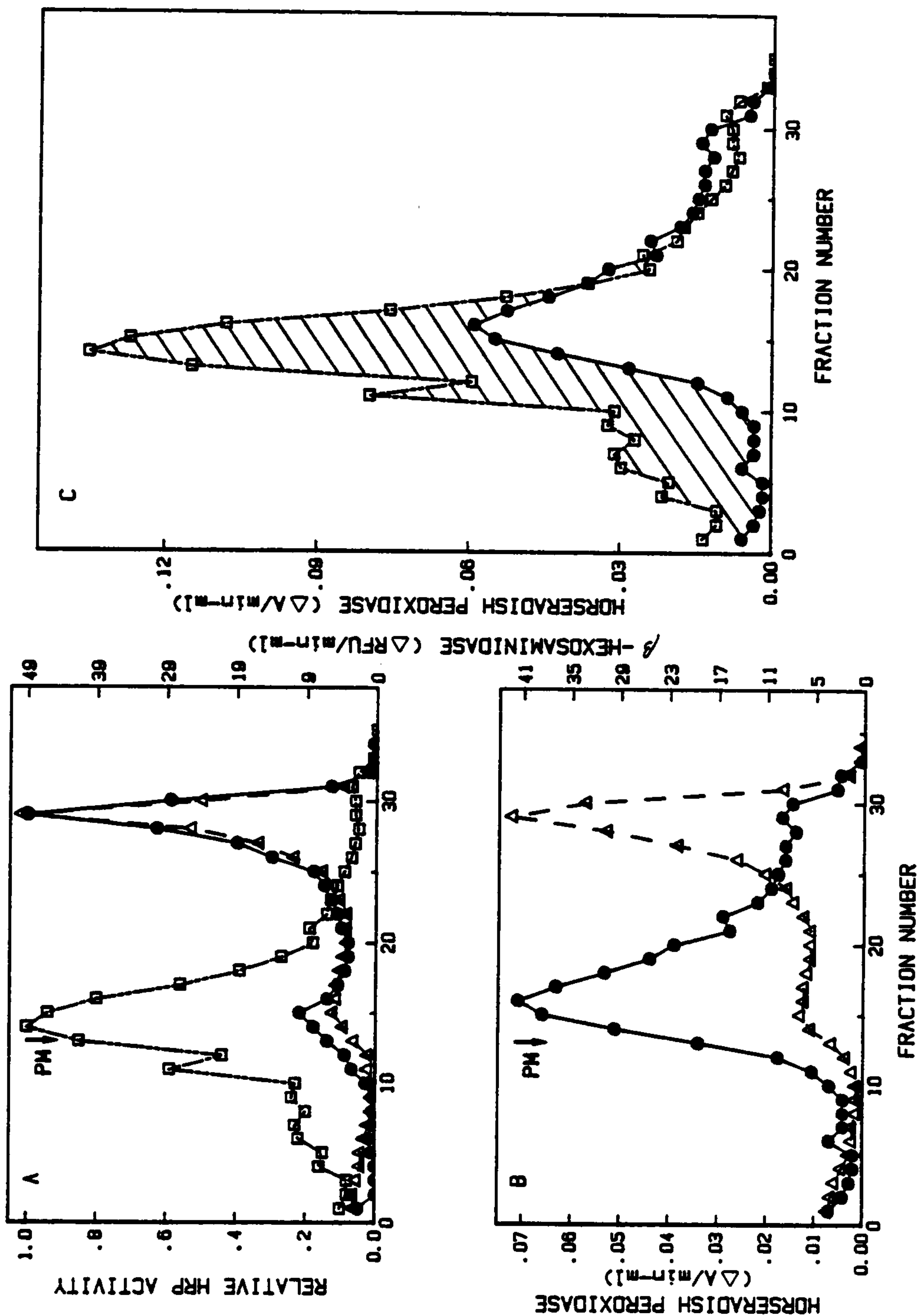
HRP, β-hexosaminidase (lysosomal marker) and alkaline phosphodiesterase I (plasma membrane marker) were assayed as described in previous work (see previous chapter, Pool et al., 1983).

Results

In all experiments described, cells were pulsed with HRP for 3 or 10 minutes at 37°C followed by a chase in HRP-free medium in the absence or presence of an inhibitory condition. Postnuclear supernatants were then fractionated in 10% Percoll density gradients (Storrie et al., 1984a). These gradients are effective in separating endocytic vesicle compartments from both plasma membrane and lysosomes (Pool et al., 1983; Storrie et al., 1984a). Figure 1A shows the HRP distribution after an uptake (3 miutes) and after a pulse-chase (3 minute pulse followed by a 45 minute chase). HRP was found initially in an "early" endocytic vesicle compartment which was slightly more dense than plasma membrane, consistant with previous results (Pool et al., 1983). A similar pattern was seen for pulses up to 10 minutes (Pool et al., 1983). For cells pulsed with HRP for 3 minutes and then chased in HRP-free media for 45 minutes at 37°C, the distribution of HRP activity in the 10% Percoll gradient was coincident with that of the lysosomal marker β-hexosaminidase as seen in previous results (Storrie et al., 1984a). Chase times of 10-15 minutes are sufficient to produce coincident distribution patterns of HRP and β-hexosaminidase (Storrie et al., 1984a).

The β-hexosaminidase distribution is shown in most

Figure 1. <u>Distribution of HRP Activity After a Pulse and Pulse-chase.</u> In A, cells were pulsed either with HRP and fractionated immediately or fractionated after a 3 minute pulse followed by a 45 minute chase at 37°C. All enzyme activity values were normalized to a peak activity of one. HRP, 3 minute pulse (□- - - -□); HRP, 3 minute pulse, 45 minute chase (●———●); alkaline phosphodiesterase I ('PM' arrow); β-hexosaminidase (△- - -△, lysosomal marker). In B, cells were pulsed with HRP for 3 minutes at 37°C chased at 17°C for 45 minutes and then fractionated. HRP (●———●); β-hexosaminidase (△- - -△). In C, is a comparison of the HRP distribution of the 3 minute pulse from A and the 17°C chase from B. The 17°C chase distribution was corrected to the pulse distribution (for differences in the efficiency of homogenization) by using the average of the ratios of alkaline phosphodiesterase I and β-hexosaminidase activities from both postnuclear supernatants and (for differences in HRP recovery) a ratio of the percent HRP recovered from both gradients.


A
B
C
PM
RELATIVE HRP ACTIVITY
HORSERADISH PEROXIDASE (∆A/min-ml)
β-HEXOSAMINIDASE (∆RFU/min-ml)
FRACTION NUMBER
0.0
.2
.4
.6
.8
1.0
0.00
.01
.02
.03
.04
.05
.06
.07
.03
.06
.09
.12
0
9
19
29
39
49
5
11
17
23
35
41
10
20
30

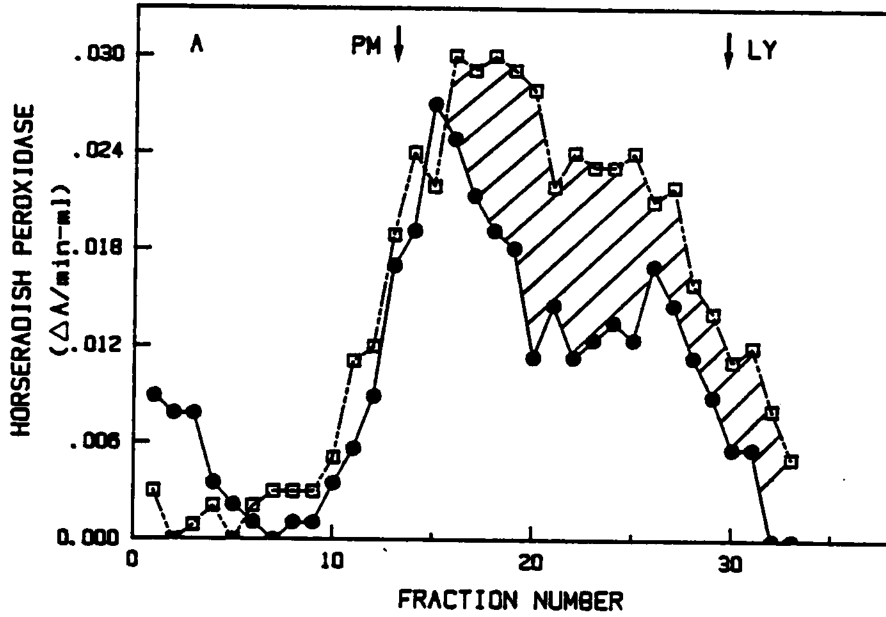
figures as a reference for HRP distribution relative to lysosomes and the effects, if any, of inhibitors on the distribution of lysosomes. The location of alkaline phosphodiesterase I (plasma membrane marker) activity in the gradient is indicated by an arrow labeled 'PM' in all figures. The distribution of alkaline phosphodiesterase I was not effected by any of the inhibitors used.

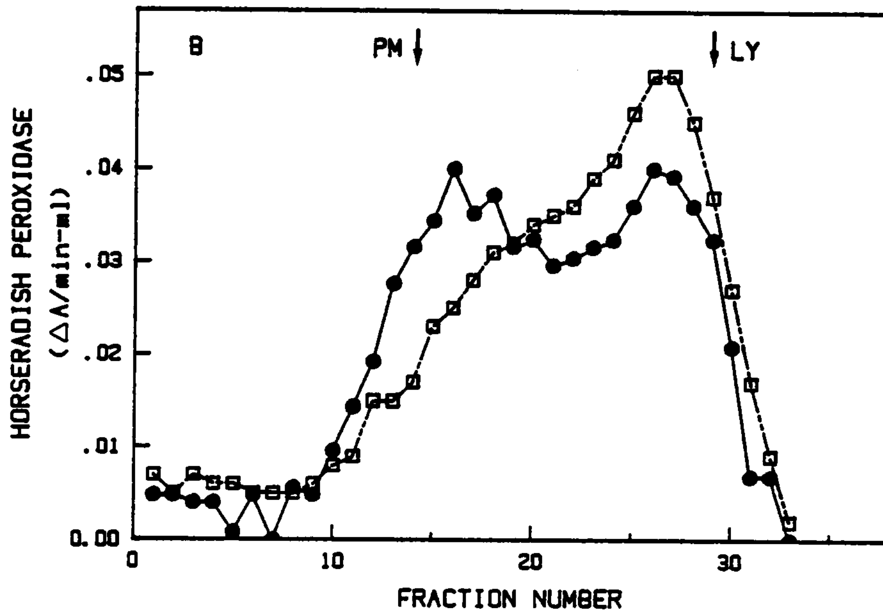
Effects of Reduced Temperature on Transport

To determine the effects of reduced temperature on the HRP transport from early endocytic vesicles, cells were pulsed with HRP for 3 minutes and then incubated for 45 minutes at 17°C (chase). Inhibition of transport into lysosomes occurred with HRP being retained predominately in a vesicle population slightly more dense than plasma membrane (an "early" endocytic vesicle compartment) (Figure 1B). Comparison of the HRP distribution from pulse labeled cells (3 minute pulse) with that from cells after a 17°C chase indicated that during the chase ~65% of the initial HRP was lost from a low density compartment (shaded area, Figure 1C) which represents "very early" endocytic vesicles (see Casey et al., 1985). This loss of HRP activity is presumably due to exocytosis. A major portion of pulse ingested HRP has been shown previously to be exocytosed during a chase (Adams et al., 1982).

We next investigated if reduced temperature strongly inhibited HRP transport from later endocytic vesicle compartments. To localize HRP in "early-intermediate" endocytic vesicles, cells were pulsed with HRP and chased for 3 minutes at 37°C (Figure 2A). With a 45 minute 17°C chase, transport to lysosomes was inhibited and the HRP distribution was found to be somewhat skewed toward "early" vesicles. Note that a ~30% decrease in total HRP activity in the gradient was observed. Most of this decrease was apparently from "intermediate" vesicles (shaded area, Figure 2A). This loss of HRP activity was presumably by exocytosis. For cells pulsed and then chased for 6 minutes at 37°C, HRP activity was localized in "intermediate-late" endocytic vesicles (Figure 2B). Following a 45 minute chase at 17°C, no transport of HRP toward lysosomes was observed and HRP was found distributed throughout "early", "intermediate" and "late" vesicles (Figure 2B). After the reduced temperature chase, no loss of total HRP activity in the gradient was observed, although a decrease in activity in "intermediate-late" endocytic vesicles with a corresponding increase in HRP activity in an "early" vesicle compartment was observed. These observations suggest that reduced temperature inhibits "forward" transport of HRP to denser vesicle compartments in all

Figure 2. <u>The Effect of Reduced Temperature on Transport Out of Endocytic Vesicles Following a 37°C Chase.</u> In A, cells were either pulsed with HRP for 3 minutes followed by a 3 minute chase at 37°C and then fractionated or fractionated after a pulse (3 minutes) and chase (3 minutes) at 37°C followed by a 45 minute chase at 17°C. The distributions following the 17°C chase were corrected as described in figure 1C. HRP, 37°C pulse and chase (□----□); HRP, 37°C pulse and chase followed by 17°C chase (•——•); alkaline phosphodiesterase I ('PM' arrow); β-hexosaminidase ('LY' arrow). In B, cells were either pulsed with HRP for 3 minutes followed by a 6 minute chase at 37°C and then fractionated or fractionated after a pulse (3 minutes) and chase (6 minutes) at 37°C followed by a 45 minute chase at 17°C. The 17°C chase distribution were corrected as described in A. HRP, 37°C pulse and chase (□----□); HRP, 37°C pulse and chase followed by 17°C chase (•——•). For control see figure 1A.


A
PM
LY
HORSERADISH PEROXIDASE
(ΔA/min-ml)
.030
.024
.018
.012
.006
0.000
0
10
20
30
FRACTION NUMBER


B
PM
LY
HORSERADISH PEROXIDASE
(ΔA/min-ml)
.05
.04
.03
.02
.01
0.00
0
10
20
30
FRACTION NUMBER

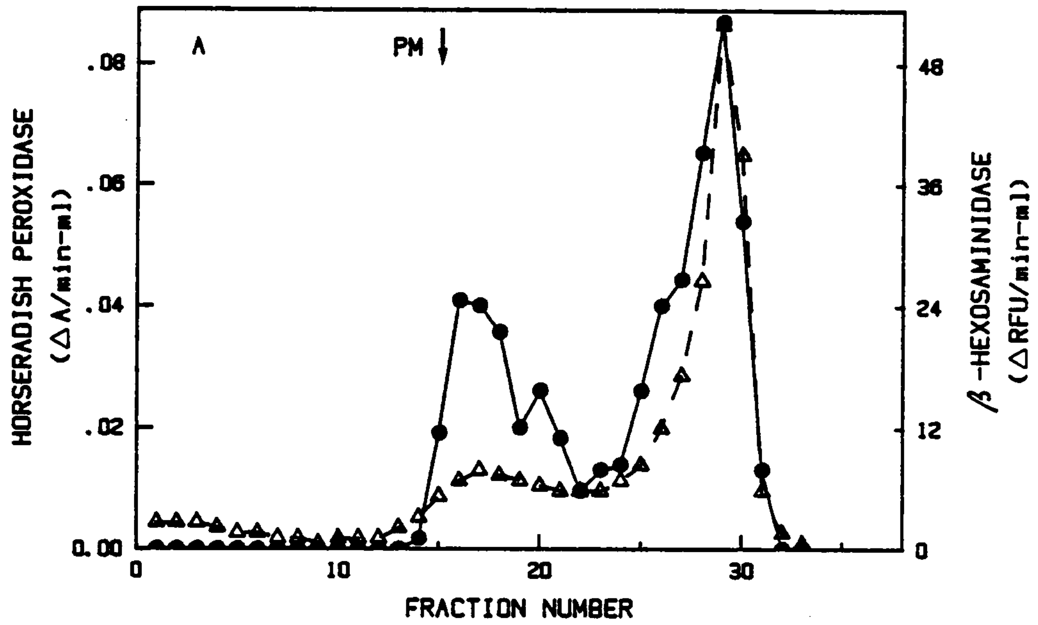
cases. Moreover, the observed decrease of HRP activity in "intermediate-late" endocytic vesicles with a corresponding increase in "early" endocytic vesicles during a 17°C chase (Figure 2B) suggests that a reverse flow from "late" to "early" vesicles can occur.

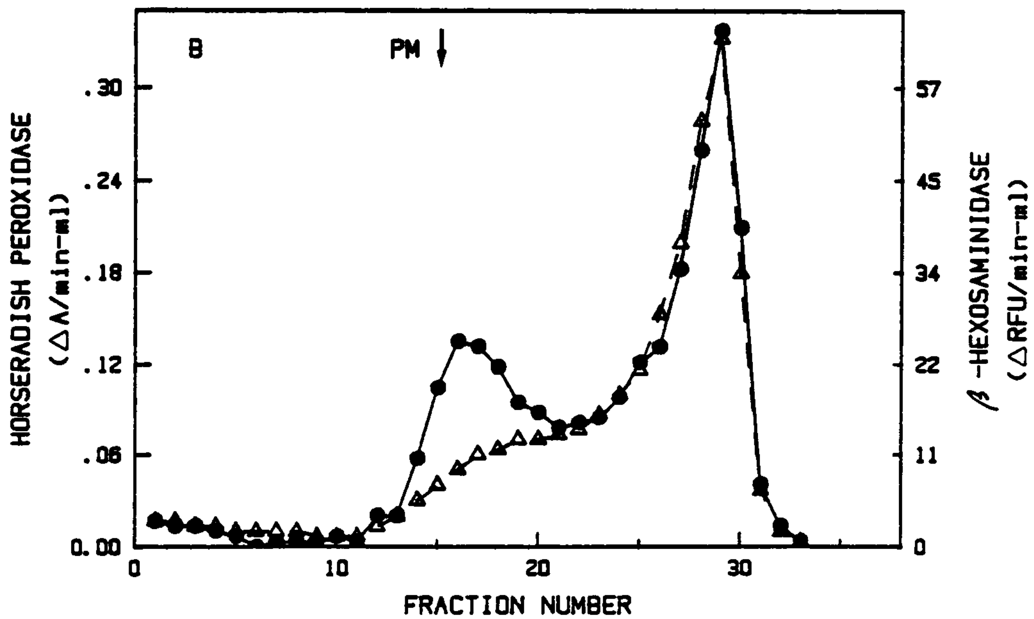
The temperature sensitivity demonstrated may represent either an absolute block or a partial inhibition of HRP transport to later vesicle compartments. To distinguish between these two possibilities, cells were incubated with HRP continuously for 6 or 18 hours at 17°C and the postnuclear supernatants were then fractionated in 10% Percoll gradients (Figure 3A and 3B). Under control conditions (i.e. continuous uptake at 37°C) HRP accumulates in lysosomes (Storrie et al., 1984b). Figure 3 shows that at 17°C, approximately two-thirds of the HRP accumulated in lysosomes while one-third was in "early" endocytic vesicles. Little to no HRP activity was seen in "intermediate" endocytic vesicles. These data suggest that reduced temperature is not an absolute block to all HRP transport and that "early" steps in transport are more sensitive to reduced temperature than are "intermediate" steps.

<u>Effects of pH Elevators on Transport</u>

It has been demonstrated that acidification of

Figure 3. The Long Term Effect of a 17°C Incubation and Distribution of HRP Between Vesicle Compartments. Cells were incubated with HRP (0.5 mg/ml) at 17°C for 6 hours, A or 18 hours, B and then fractionated. HRP, (●——●); alkaline phosphodiesterase I ('PM' arrow); β -hexosaminidase (△– – –△). For control see figure 1A.

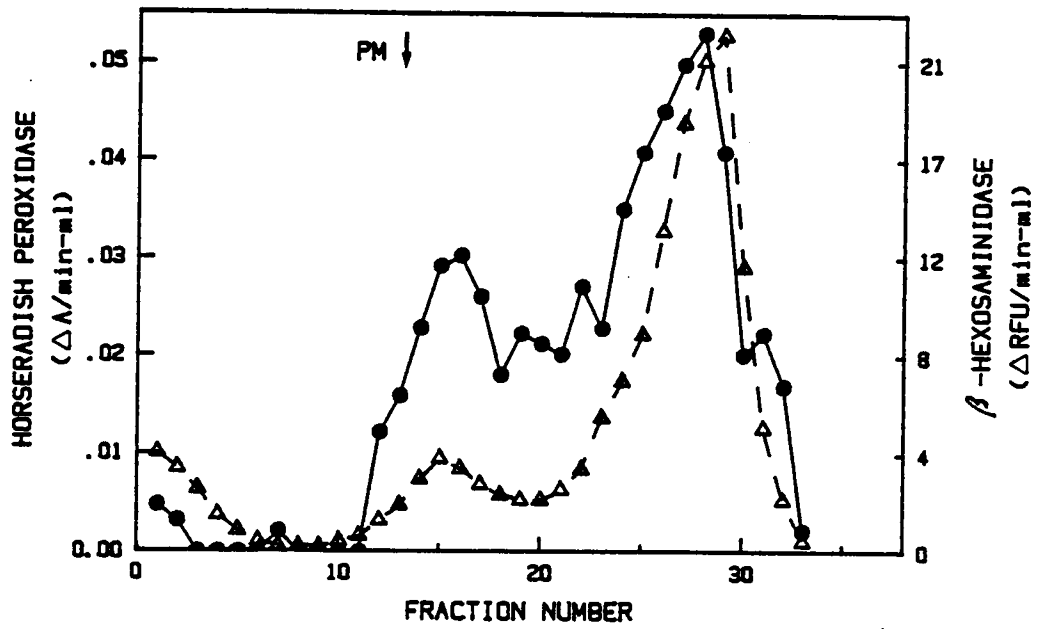

A
PM
HORSERADISH PEROXIDASE
(ΔA/min-ml)
β-HEXOSAMINIDASE
(ΔRFU/min-ml)
FRACTION NUMBER
0.00
.02
.04
.06
.08
0
12
24
36
48
0
10
20
30


B
PM
HORSERADISH PEROXIDASE
(ΔA/min-ml)
β-HEXOSAMINIDASE
(ΔRFU/min-ml)
FRACTION NUMBER
0.00
.06
.12
.18
.24
.30
0
11
22
34
45
57
0
10
20
30

endocytic vesicles occurs rapidly following endocytosis (Tycko and Maxfield, 1982; Galloway et al., 1983). Many substances have been used to raise intravesicular pH including NH_4Cl (Ohkuma and Poole, 1978; Poole and Ohkuma, 1981; Galloway et al., 1983) methylamine (Ohkuma and Poole, 1978; Poole and Ohkuma, 1981; Maxfield, 1982), chloroquine (Ohkuma and Poole, 1978; Poole and Ohkuma, 1981; Maxfield, 1982; Murphy et al., 1984) and monensin (Maxfield, 1982). These pH elevators can be divided into two classes based on their mode of action. NH_4Cl, methylamine and chloroquine are all amines which accumulate in acidic compartments. Monensin is a carboxylic ionophore which collapses proton gradients by a one-to-one exchange of protons and monovalent cations (preferentially Na^+) across membranes (Tartakoff, 1983).

To test the effect of pH elevators on HRP transport, cells were pulsed with HRP for 10 minutes and chased for 15 minutes in HRP-free medium in the presence of 15 mM NH_4Cl or 10 μM monensin. In the presence of NH_4Cl, HRP transport during the chase was inhibited (Figure 4, versus Figure 1A). HRP was localized in "early", "intermediate" and "late" endocytic vesicles and probably lysosomes. The major peak of HRP activity corresponded to the position of "late" endocytic vesicles and lysosomes in the gradient. A smaller peak at "early" vesicles was also observed.

Figure 4. <u>The Effect of NH_4Cl on HRP Transport to Lysosomes.</u> Cells were pulsed with HRP (0.5 mg/ml) for 10 minutes at 37°C and then chased for 15 minutes at 37°C in the presence of 15 mM NH_4Cl followed by fractionation. HRP, (●——●); alkaline phosphodiesterase I ('PM' arrow); β-hexosaminidase, (△- - -△). For control see figure 1A.



PM
HORSERADISH PEROXIDASE (ΔA/min-ml)
β-HEXOSAMINIDASE (ΔRFU/min-ml)
FRACTION NUMBER
0.00
.01
.02
.03
.04
.05
0
4
8
12
17
21
0
10
20
30

NH_4Cl also had little, if any, effect on the distribution of lysosomes (Figure 4). In the presence of monensin, HRP after the chase was localized in "early-late" endocytic vesicles and lysosomes (Figure 5). A slight peak of HRP activity was seen at "early" endocytic vesicles. It should also be noted that in the presence of monensin, the β-hexosaminidase activity in the gradient was skewed toward a lighter density (Figure 5). This skewing may be caused by swelling of lysosomes, an acidic compartment. Elements of the Golgi apparatus have been observed to swell in the presence of monensin (Tartakoff, 1983; Devault et al., 1984). Together, these data suggest "early", "intermediate" and "late" steps in HRP transport are pH sensitive and that "early" step(s) may be somewhat more sensitive than later steps.

We have investigated the effects of the common media supplement HEPES (N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid) upon HRP transport into lysosomes. Cells were pulsed with HRP for 10 minutes in the presence of 20 mM HEPES (pH 7.2) and then chased for 10 minutes (Figure 6A) or 15 minutes (Figure 6B). Following a 10 minute chase, HRP activity was localized in "early", "intermediate" and "late" endocytic vesicles and probably lysosomes. With a longer chase, HRP activity was shifted more toward "intermediate-late" endocytic vesicles and

Figure 5. The Effect of Monensin on HRP Transport to Lysosomes. Cells were pulsed with HRP (0.5 mg/ml) for 10 minutes at 37°C and then chased for 10 minutes at 37°C in the presence of 10 μM monensin followed by fractionation. HRP, (•———•); alkaline phosphodiesterase I ('PM' arrow); β-hexosaminidase, (△– – –△). For control see figure 1A.

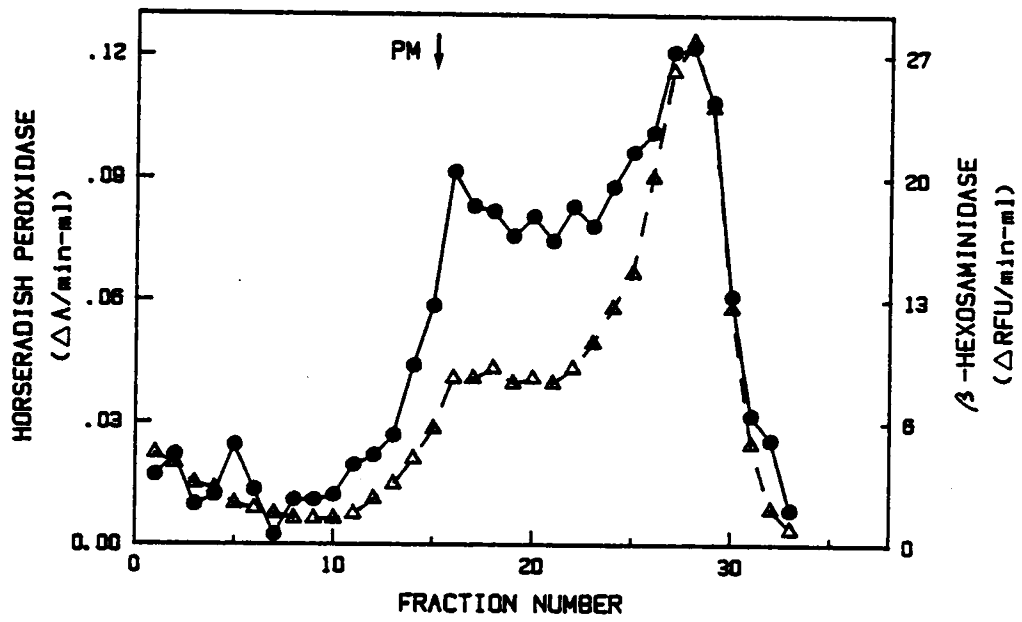
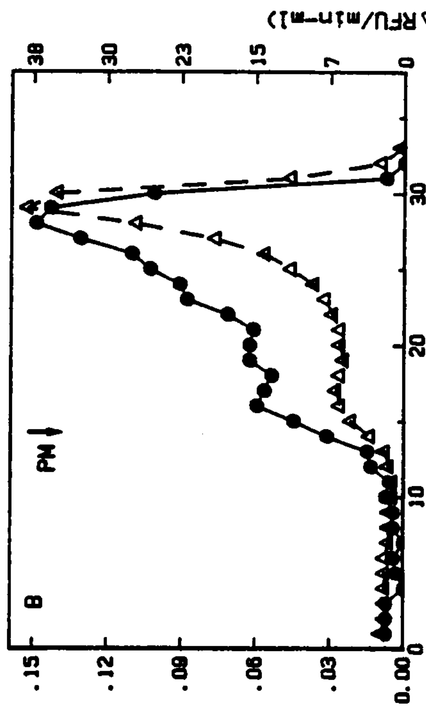
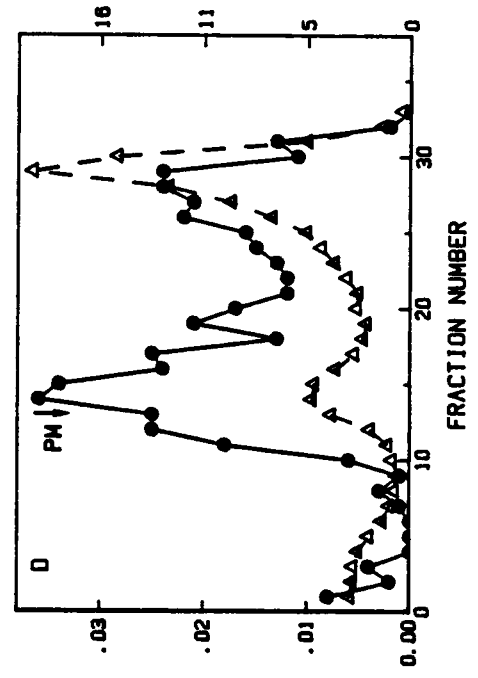
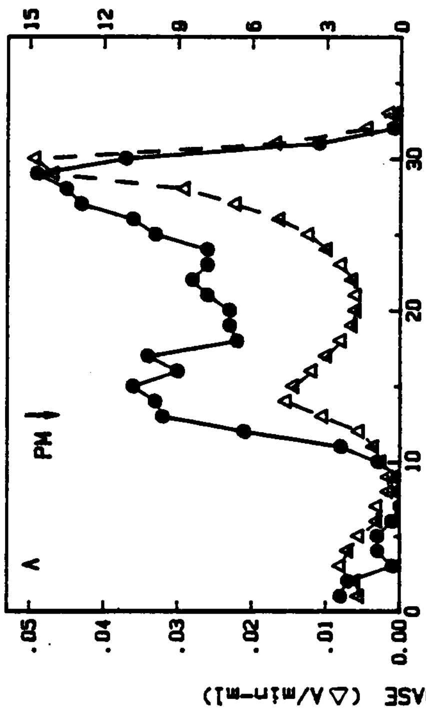
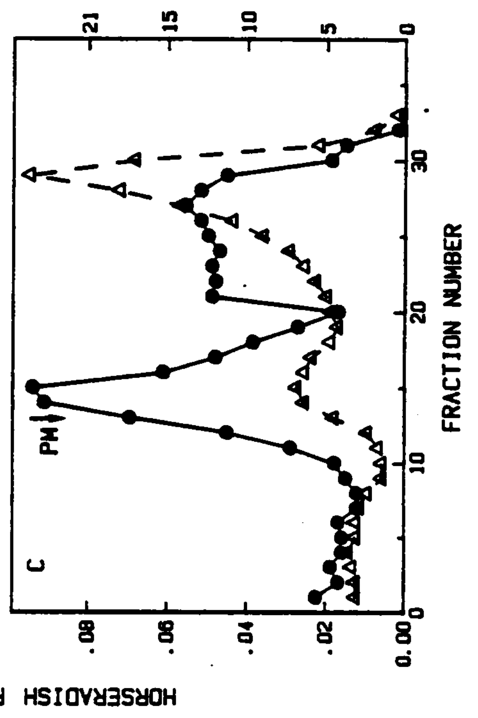

PM
.12
.09
.06
.03
0.00
HORSERADISH PEROXIDASE
(ΔA/min-ml)
27
20
13
6
0
β-HEXOSAMINIDASE
(ΔRFU/min-ml)
0
10
20
30
FRACTION NUMBER

Figure 6. <u>The Effect of HEPES on HRP Transport to Lysosomes.</u> In A and B, cells were pulsed with HRP (0.5 mg/ml) for 10 minutes at 37°C in the presence of 20 mM HEPES and then chased for 10 minutes, A or 15 minutes, B at 37°C in the presence of 20 mM HEPES followed by fractionation. In C and D, cells were pulsed with HRP (0.5 mg/ml) for 10 minutes at 37°C in the presence of 40 mM HEPES and then chased for 10 minutes, C or 15 minutes, D at 37°C in the presence of 40 mM HEPES followed by fractionation. HRP, (●———●); alkaline phosphodiesterase I ('PM' arrow); β-hexosaminidase, (△– – –△). For control see figure 1A.


A
PM
HORSERADISH PEROXIDASE (ΔA/min-ml)


B
PM
β-HEXOSAMINIDASE (ΔRFU/min-ml)


C
PM
FRACTION NUMBER

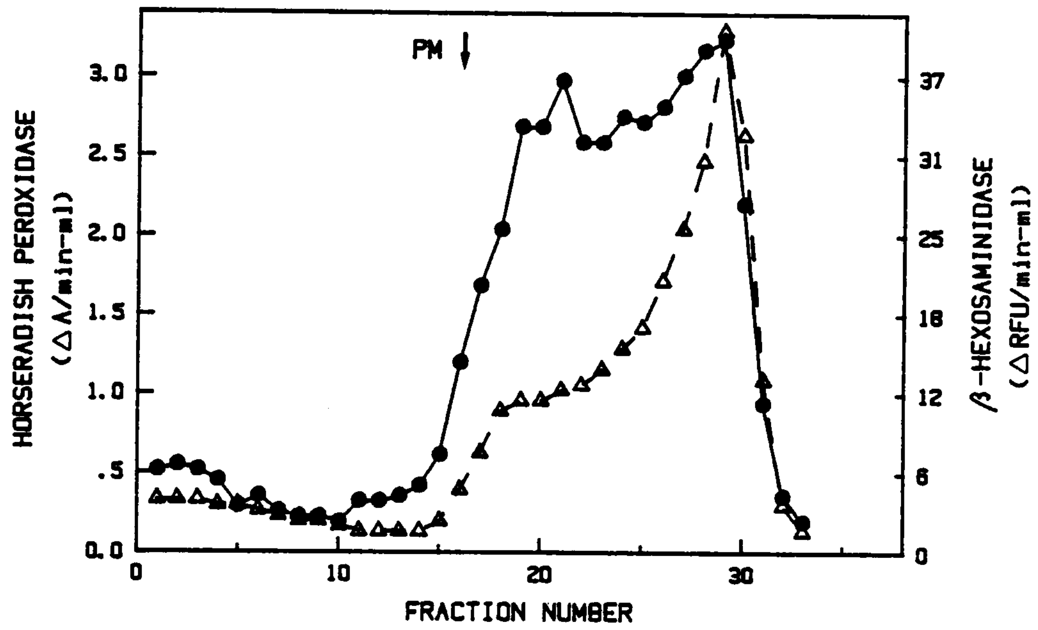

D
PM
FRACTION NUMBER

lysosomes. Again, these results suggest a pH dependence of transport. Increasing the concentration of HEPES to 40 mM (pH 7.2) gave a stronger inhibition of transport (Figure 6C and 6D). It should be noted that the β-hexosaminidase distribution is not perturbed by the presence of HEPES. To test the long term effect of HEPES on HRP transport through endocytic vesicles, cells were incubated with HRP for 6 hours in the presence of 20 mM HEPES (Figure 7). HRP activity after this long term uptake was distributed predominately in "intermediate-late" endocytic vesicles and lysosomes with a small portion in "early" vesicles. The β-hexosaminidase distribution was skewed slightly toward a lighter density. Taken together, the HEPES data suggest "early", "intermediate" and "late" steps in transport are pH sensitive and that "early" step(s) are more sensitive than later steps. It should be noted that none of these putative pH elevators inhibits HRP dissociation from endocytic vesicle membrane (see chapter II).

<u>Effects of Energy Inhibitors on Transport</u>

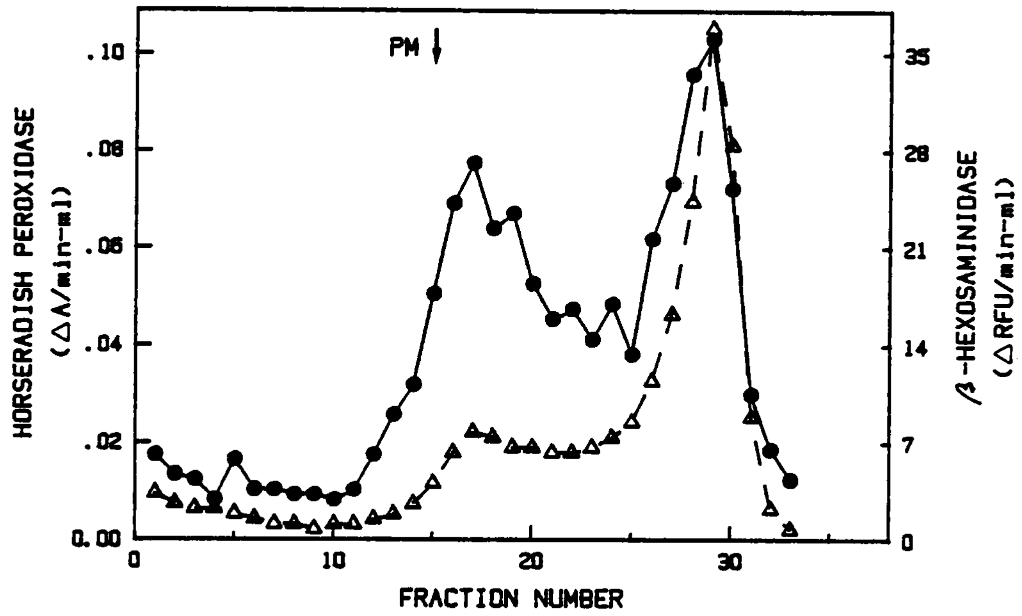
To test the effects of ATP depletion on HRP transport, cells were pulsed with HRP for 10 minutes and then chased for 45 minutes in the presence of 2 mM NaF and 1 mM KCN (Figure 8). NaF was used to inhibit glycolysis

Figure 7. The Long Term Effect of HEPES on HRP Transport to Lysosomes. Cells were incubated with HRP (0.5 mg/ml) at 37°C for 6 hours in the presence of 20 mM HEPES and then fractionated. HRP, (●——●); alkaline phosphodiesterase I ('PM' arrow); β-hexosaminidase, (△- - -△) For control see figure 1A.



PM
3.0
2.5
2.0
1.5
1.0
.5
0.0
HORSERADISH PEROXIDASE
(ΔA/min-ml)
37
31
25
18
12
6
0
β-HEXOSAMINIDASE
(ΔRFU/min-ml)
0
10
20
30
FRACTION NUMBER

Figure 8. The Effect of NaF/KCN on HRP Transport to Lysosomes. Cells were pulsed with HRP (0.5 mg/ml) for 10 minutes at 37°C and then chased for 45 minutes at 37°C in the presence of 2 mM NaF and 1 mM KCN followed by fractionation. HRP, (●———●); alkaline phosphodiesterase I ('PM' arrow); β-hexosaminidase, (△– – –△). For control see figure 1A.


PM
HORSERADISH PEROXIDASE
(ΔA/min-ml)
β-HEXOSAMINIDASE
(ΔRFU/min-ml)
FRACTION NUMBER
.10
.08
.06
.04
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0.00
35
28
21
14
7
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10
20
30

at enolase and KCN to inhibit oxidative phosphorylation at cytochrome oxidase. HRP distribution after the chase was predominately in "early" and "late" endocytic vesicles and to a lesser extent, in "intermediate" vesicles. This effect may be either a direct consequence of energy requirements or an indirect effect on vesicle pH.

Discussion

Horseradish peroxidase (HRP) is internalized as a solute which binds to endocytic vesicle membrane and then dissociates to accumulate in lysosomes as a solute. This represents a unique system of endocytic transport. We have used reduced temperature, reagents which elevate vesicular pH and inhibitors of ATP production to inhibit intracellular transport of HRP and to provide evidence for the mechanisms which underlie this transport process. A minimum of four steps in this transport process have been identified based on sensitivity to the various inhibitors. These include transport steps through progressively denser "very early", "early", "intermediate" and "late" endocytic vesicle compartments.

Reduced temperature (17°C) appears to differentially inhibit HRP transport into lysosomes versus HRP exocytosis and "reverse" flow through endocytic vesicles. HRP transport into lysosomes is strongly inhibited at 17°C irrespective of the endocytic vesicle population in which peroxidase is present at the start of the 17°C chase. However, "reverse" flow of HRP is readily detectable from all endocytic vesicle compartments at reduced temperature. The differential loss of HRP from the light density portion of a pulse distribution suggests the existance of a "very early" endocytic vesicle compartment. Such a

"very early" compartment has been identified by a cell fractionation approach (Casey et al., 1985). "Reverse" flow appears to be a property of all endocytic vesicle populations, although exocytosis may occur preferentially from a low density endocytic compartment.

The long term transport of HRP into lysosomes at 17°C demonstrates that inhibition of transport by reduced temperature does not constitute an absolute block. Dunn et al. (1980) have reported that, for receptor-mediated endocytosis (asialoglycoprotein receptor), temperatures below 20°C completely inhibit transport into lysosomes. The source of the apparent difference between our results and those of Dunn et al. (1980) may relate to differences in the transport system studied. The pronounced accumulation of HRP in an "early" endocytic compartment versus later (i.e. "intermediate" and "late") compartments, during a long term 17°C uptake, suggests that reduced temperature exerts a stronger effect on "early" transport steps then later ones. The basis for an inhibition of transport at "early" steps may be due to the inability of HRP to dissociate from vesicle membrane at 17°C. It has been demonstrated that HRP does not dissociate from endocytic vesicle membrane at 17°C over a 30 minute chase period (see chapter II). Inhibition of transport at later (i.e. "intermediate" and "late") steps,

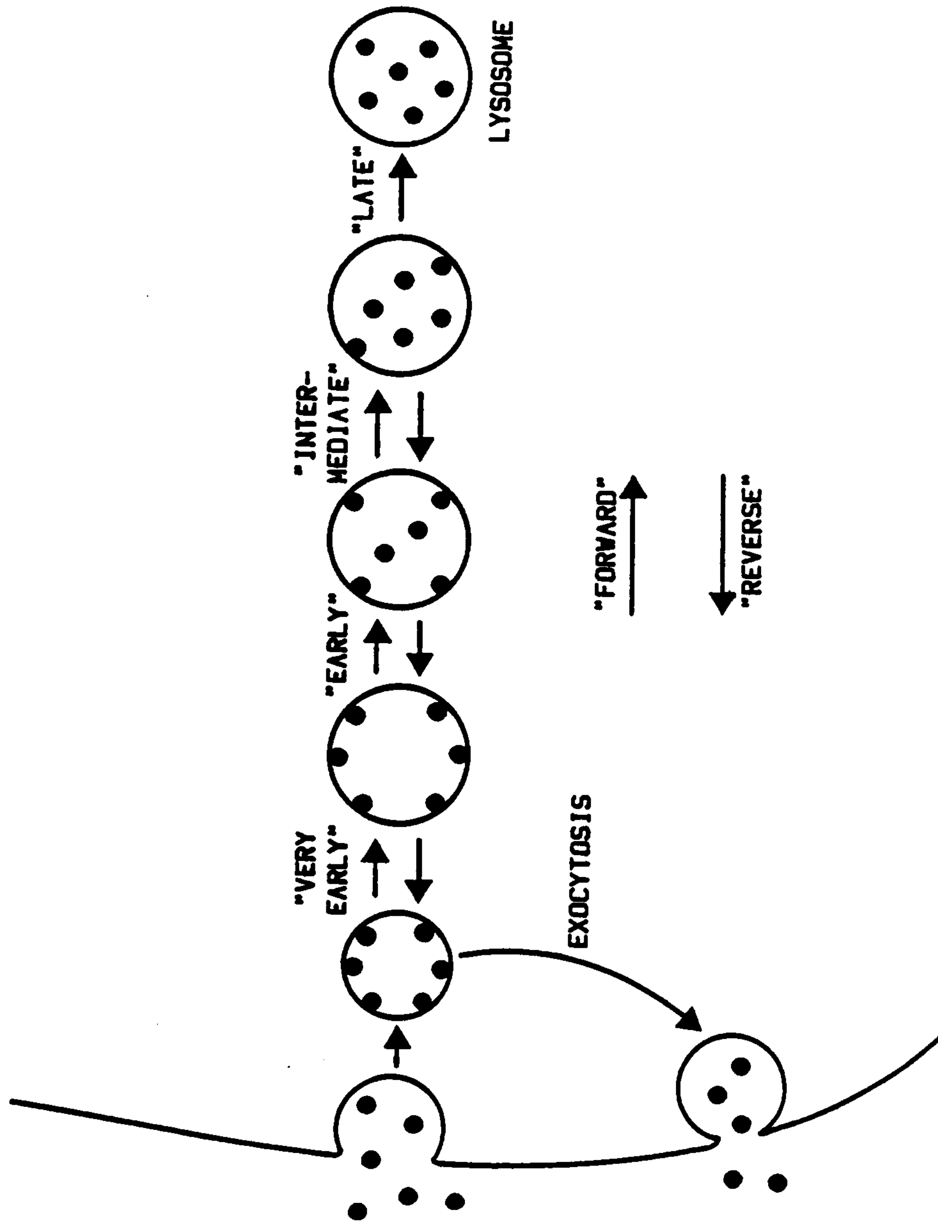
appears to be independent of HRP-membrane dissociation. HRP in these compartments is significantly dissociated from the membrane. Wolkoff et al. (1984) have also concluded that reduced temperature effects an "early" receptor-ligand dissociation step as well as later step(s) following receptor-ligand dissociation, in an asialoglycoprotein receptor system.

The inhibition of HRP transport by pH elevators (NH_4Cl, monensin, HEPES) is likely independent of HRP-membrane dissociation. Both monensin and HEPES fail to inhibit the dissociation of HRP from membrane (see chapter II). NH_4Cl and monensin also fail to block exocytosis (Pool and Storrie; Casey and Storrie, unpublished observations). This suggests again that exocytosis is less sensitive to inhibitors than transport to lysosomes. The pH elevators, as with reduced temperature, appeared to give the strongest inhibition of "forward" transport at the level of "early" endocytic vesicles. In the presence of monensin, the increased accumulation of HRP in an "intermediate" compartment, as compared to that seen with the other pH elevators, may indicate "intermediate" step(s) which are particularily sensitive to monensin. Elevation of vesicle pH may alter the ability of transport vesicles to fuse with lysosomes through a transmembrane effect. Using NaF/KCN, which

should lower cellular ATP, similar effects are seen to those of the pH elevators. This inhibition may be due to a direct role on an ATP dependent process or a secondary effect of vesicle pH elevation through an inhibition of an ATP requiring proton pump.

It should be emphasized that the inhibitory effect on transport demonstrated by HEPES indicates a note of caution in using HEPES, and possibly other buffering media supplements, in studies of transport phenomena. Figure 9 summarizes the steps in HRP transport apparent from these studies.

Figure 9. <u>The Proposed Steps Involved in the Uptake and Intracellular Transport of HRP.</u>


"VERY
EARLY"
"EARLY"
"INTER-
MEDIATE"
"LATE"
LYSOSOME
EXOCYTOSIS
"FORWARD"
"REVERSE"

References

Adams, C.J., Maurey, K.M., Storrie, B. (1982) Exocytosis of pinocytic contents by Chinese hamster ovary cells. J. Cell Biol., 93:632-637.

Anderson, R.G.W., Brown, M.S., Beisiegel, U., Goldstein, J.L. (1982) Surface distribution and recycling of the low density lipoprotein receptor as visualized with antireceptor antibodies. J. Cell Biol., 93:523-531.

Casey, K.A., Maurey, K.M., Storrie, B. (1985) Fluid phase pinocytosis in fibroblasts: Characterization of early compartments. (manuscript in preparation).

Devault, A., Zollinger, M., Crine, P. (1984) Effects of the monovalent ionophore monensin on the intracellular transport and processing of pro-opiomelanocortin in cultured intermediate lobe cells of the rat pituitary. J. Biol. Chem., 259:5146-5151.

Dunn, W.A., Hubbard, A.L., Aronson, N.N.Jr. (1980) Low temperature selectively inhibits fusion between pinocytic vesicles and lysosomes during heterophagy of ^{125}I-asialofetuin by the perfused rat liver. J. Biol. Chem., 255:5971-5978.

Galloway, C.J., Dean, G.E., Marsh, M., Rudnick, G., Mellman, I. (1983) Acidification of macrophage and fibroblast endocytic vesicles in vitro. Proc. Natl. Acad. Sci. U.S.A. 80:3334-3338.

Gonzalez-Noriega, A., Grubb, J.H., Talkad, V., Sly, W.S. (1980) Chloroquine inhibits lysosomal enzyme pinocytosis and enhances lysosomal enzyme secretion by impairing receptor recycling. J. Cell Biol., 85:839-852.

Harford, J., Wolkoff, A.W., Ashwell, G., Klausner, R.D. (1983) Monensin inhibits intracellular dissociation of asialoglycoproteins from their receptor. J. Cell Biol., 96:1824-1828.

Maxfield, F.R. (1982) Weak bases and ionophores rapidly and reversibly raise the pH of endocytic vesicles in cultured mouse fibroblasts. J. Cell Biol., 95:676-681.

Merion, M., Schlesinger, P., Brooks, R.M., Moehring, J.M., Moehring, T.J., Sly, W.S. (1983) Defective acidification of endosomes in Chinese hamster ovary cell mutants "cross-resistant" to toxins and viruses. Proc. Natl. Acad. Sci.

U.S.A., 80:5315-5319.

Merion, M., Sly, W.S. (1983) The role of intermediate vesicles in the adsorptive endocytosis and transport of ligand to lysosomes by human fibroblasts. J. Cell Biol., 96:644-650.

Murphy, R.F., Powers, S., Cantor, C.R. (1984) Endosome pH measured in single cell by dual fluorescence flow cytometry: Rapid acidification of insulin to pH 6. J. Cell Biol., 98:1757-1762.

Ohkuma, S. and Poole, B. (1978) Fluorescence probe measurement of the intralysosomal pH in living cells and the perturbation of pH by various agents. Proc. Natl. Acad. Sci. U.S.A., 75:3327-3331.

Pool, R.R., Maurey, K.M., Storrie, B. (1983) Characterization of pinocytic vesicles from CHO cells: Resolution of pinosomes from lysosomes by analytical centrifugation. Cell Biol. Int. Rep., 7:361-367.

Poole, B. and Ohkuma, S. (1981) Effect of weak bases on the intralysosomal pH in mouse peritoneal macrophages. J. Cell Biol., 90:665-669.

Schwartz, A.L., Bolognesi, A., Fridovich, S.E. (1984) Recycling of the asialoglycoprotein receptor and the effect of lysosomotropic amines in hepatoma cells. J. Cell Biol., 98:732-738.

Steinman, R.M., Brodie, S.E., Cohn, Z.A. (1976) Membrane flow during pinocytosis. Stereologic analysis. J. Cell Biol., 68:665-687.

Storrie, B., Pool, R.R.Jr., Sachdeva, M., Maurey, K.M., Oliver, C. (1984a) Evidence for both prelysosomal and lysosomal intermediates in endocytic pathways. J. Cell Biol., 98:108-115.

Storrie, B., Sachdeva, M., Viers, V.S. (1984b) Chinese hamster ovary cell lysosomes retain pinocytized horseradish peroxidase and in situ-radioiodinated proteins. Mol. Cell. Biol., 4:296-301.

Tartakoff, A.M. (1983) Perturbation of vesicular traffic with the carboxylic ionophore monensin. Cell, 32:1026-1028.

Tycko, B. and Maxfield, F.R. (1982) Rapid acidification of endocytic vesicles containing α-$_2$-macroglobulin. Cell, 28:643-651.

Wolkoff, A.W., Klausner, R.D., Ashwell, G., Harford, J. (1984) Intracellular segregation of asialoglycoproteins and their receptor: A prelysosomal event subsequent to dissociation of the ligand-receptor complex. J. Cell Biol., 98:375-381.

Chapter IV

General Summary and Concluding Remarks

The endocytosis of horseradish peroxidase (HRP) in this cell line demonstrates a unique endocytic transport system. HRP, internalized as a solute, once in endocytic vesicles is reversibly bound to site(s) on the luminal face of vesicle membrane. This binding site(s) appears to be unique to endocytic vesicles. Dissociation of HRP from binding site(s), a temperature dependent but pH independent process, occurs as HRP is transported to lysosomes through a minimum of four steps ("very early", "early", "intermediate" and "late"). HRP in lysosomes is no longer associated with membrane.

Transport of HRP through endocytic vesicles is able to occur in both a "forward" (i.e. from "very early" compartment(s) to lysosomes) and "reverse" (i.e. from "late" compartment(s) to "very early" compartment(s)) direction. All steps in the "forward" flow of HRP appear to be inhibited by reduced temperature and elevation of vesicle pH/ATP depletion and generally appear more sensitive to reduced temperature then "reverse" flow. The reduced temperature inhibition of transport is preferential for "early" steps and may be due to an inhibition of HRP-membrane dissociation. Transport

inhibition by reduced temperature at later steps is independent of HRP dissociation and may occur through a decrease in membrane fluidity, inhibiting vesicle-vesicle/vesicle-lysosome fusion. However reduced temperature inhibition of transport is not an absolute block. Exocytosis of HRP, likely occurring preferentialy from a "very early" compartment(s) is also not blocked at a temperature which inhibits transport. Inhibition of transport by elevation of vesicle pH/ATP depletion, like reduced temperature, occurs at each step in the "forward" flow of HRP but is preferential for "early" and "late" steps. Monensin, a pH elevator, may also strongly inhibit transport at "intermediate" step(s). Elevation of vesicle pH does not inhibit the dissociation of HRP from vesicle membrane therefore, it may be suggested that inhibition of transport is due to a pH dependent transmembrane effect.

Speculating on the uniqueness, to this system, of the HRP binding site(s), it should be pointed out that HRP appears to be internalized in this system as a fluid molecule and would likely enter vesicles, at least initially, common to both a fluid phase endocytosis and HRP transport system. Therefore site(s) on endocytic vesicle membrane which bind HRP may be present in all endocytic vesicles. The unique nature of the HRP binding

site(s) to endocytic vesicle membrane, not being found on plasma membrane or in lysosomes, may suggest an intracellular pool of membrane which is exclusively used for endocytic vesicles. It is unlikely that newly synthesized membrane is continuously being fed into endocytic pathways. Therefore, a model may be proposed in which HRP and possibly true fluid phase molecules are internalized into initial vesicles derived from plasma membrane. The vesicle contents would then very rapidly be transfered to vesicles derived from an intracellular membrane pool and transported to lysosomes. Endocytic vesicle membrane would then be recycled within the cell back to the pool. The large degree of exocytosis seen in this and other systems may be due to an inefficient transfer from initial vesicles to those from the intracellular membrane pool.

One area for further effort might be the use of transport inhibitors in a true fluid phase endocytic system (eg. lucifer yellow uptake). Differences in inhibition between a fluid phase system and the HRP system may signal the presence of different transport pathways. Another area to explore may be the use of enriched preparations of endocytic vesicles, perhaps from a series of density gradients, for making monoclonal antibodies to unique endocytic vesicle markers (eg. HRP binding site(s))

These antibodies, could then be used to determine the location and flow of endocytic membrane in the cell. Monoclonal antibodies, if directed toward antigenic site(s) on the vesicle exterior, may also be used in an endocytic vesicle purification scheme.

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