Coated pits, endosome CURLing and intracellular receptor–ligand trafficking

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In mediating endocytosis of extracellular macromolecules; the major mechanism in which cells ingest nutrients, degrade hormones and maintain the protein and lipid compositions of their organelle membrane, the cell surface receptors encounter 'coated pits', migrate continuously from one organelle to another, deliver the 'cargo' and often recycle back to the cell surface. This article is an attempt to give an account of the recent advances in our understanding of the molecular events involved in the 'round trip itinerary' of cell surface receptors.

ONE of the challenges of contemporary cell biology is to unravel how the extracellular materials are taken in by the cell in a very specific manner and are transported to different destinations more often vectorially. The concept of receptors; specialized cell-surface molecules anchored on the lipid bilayer, explained how recognition and binding of ligands, viz. hormones, growth factors, and carrier molecules; such as transferrin, low-density lipoprotein (LDL), are brought about effectively. Receptors are usually proteins as often are ligands. Transferrin, an iron-carrying protein, has 50,000 such receptors on a normal hepatocyte, the liver cell[1]. The same hepatocyte contains more than 500,000 asialoglyco-protein receptors, approximately 87% of it displayed on the cell surface at steady state[2]. Once the ligand binds to a receptor, the receptor–ligand (R–L) complex gets in to the cell interior in lipid vesicles (receptor-mediated endocytosis), undergoes an intricate process of receptor–ligand dissociation, sorting, and differential transport of receptor and ligand. A plethora of research papers that appeared from the early eighties to date led to the revelation of different steps involved in these processes. Lateral mobility of R–L complexes into a specialized area called 'coated pits' on the cell membrane (small membrane invagination coated with specialized proteins), clustering of R–L complexes in coated pits, dissociation of R–L complexes in acidic endosome upon endocytosis, and subsequent seggregation of receptor and ligand in a population of endosomes with tubular out growths designated as CURL (compartment of uncoupling of receptor and ligand) for differential transport of receptor and ligand, are the prominent steps that stand out from others.

Lateral mobility of R–L complexes into coated pits

Because most ligands initially bind to receptors that are located outside the coated pits on the cell membrane, there must be a mechanism to move R–L complexes into coated pits where they cluster. Understanding the lateral diffusion of different components of fluid membrane served a prelude to understanding the parameters that affect the lateral diffusion of R–L complexes (see Figure 1). The lateral diffusion of lipids and proteins on the membrane is quantified by fluorescence redistribution after photobleaching (FRAP) technique. The method essentially involves labelling the molecule of interest with a fluorescent probe in a model membrane or in an intact cell, bleaching in one spot ($\sim 1\ \mu m$ area) on the uniformly labelled membrane using an intense laser beam, and monitoring the increase in fluorescence from that spot as a function of time. The rate of recovery is a direct measure of the

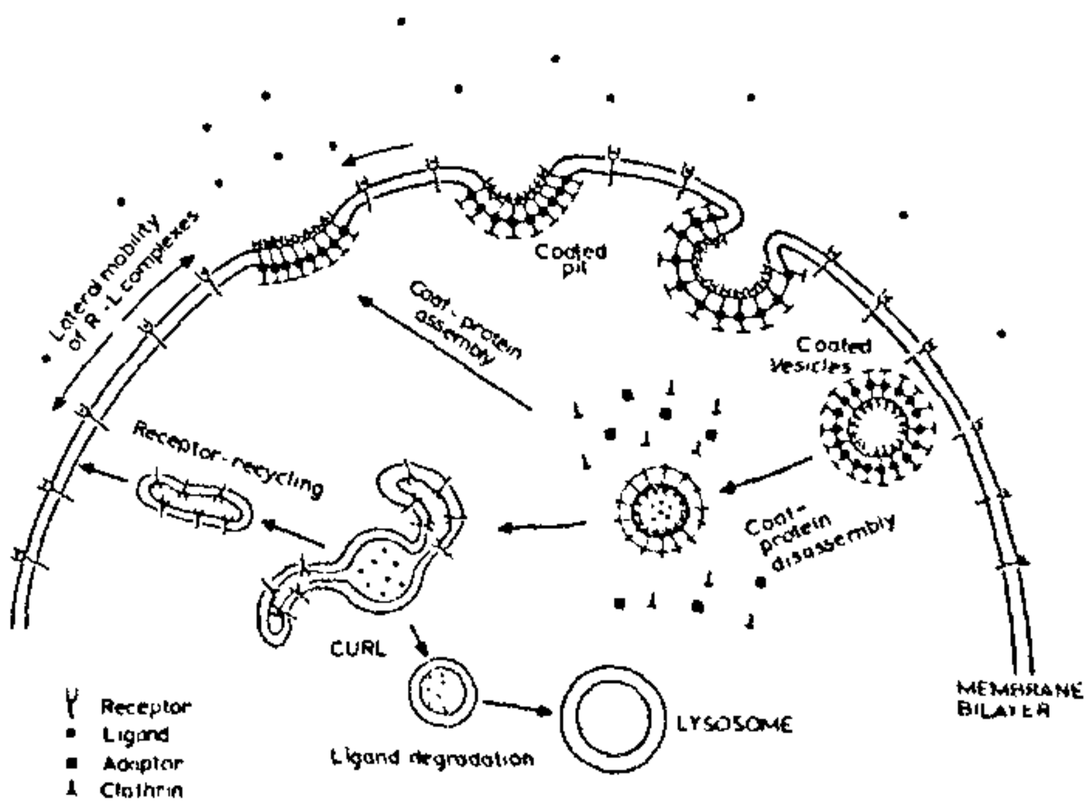

Figure 1. A pictorial representation of the different steps in the receptor-mediated endocytosis and receptor recycling (not drawn to scale) Receptors bind extracellular ligand molecules and are trapped in coated pits and *internalized*. Receptor ligand sorting occurs intracellularly in the endosome. The receptor is typically recycled back to the cell surface and the ligand is delivered to lysosomes for degradation. (Adapted from ref 16)

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lateral diffusion of the fluorescent species from the surrounding into the spot[3]. Diffusion coefficients (D) obtained for lipids in membrane from such studies were about $10^{-8}\,cm^2\,sec^{-1}$. D determined for IgE receptor, on the other hand was $3 \times 10^{-10}\,cm^2\,sec^{-1}$, almost two orders of magnitude less compared to that of lipids. The value of $10^{-12}\,cm^2\,sec^{-1}$ is considered to be effectively immobile in these experiments. The fact that the experimental lateral mobility of protein is significantly at variance with that calculated theoretically, compared to that of lipids[4], has led to the postulate that protein lateral mobility on biological membrane does not depend merely on the viscosity of the bilayer but also on other biochemical factors. This was further confirmed by comparing the Ds obtained for a variety of receptors in phospholipid vesicles (liposomes) with that obtained in natural membranes. Taking these points into consideration several workers looked into the role of intracellular membrane scaffolding often termed as cytoskeleton, cytosolic matrix or membrane matrix, in determining the lateral mobility of proteins on the cell membrane. One of such studies implicated spectrin (a matrix protein) in the mobility of band-3, an anion-transporter protein present on the erythrocyte membrane[5]. Moreover, it was revealed that acetylcholine receptor, an integral membrane protein located at the synaptic connections is immobile ($D \approx 3 \times 10^{-12}\,cm^2\,sec^{-1}$) and can be mobilized ($D \approx 3 \times 10^{-9}\,cm^2\,sec^{-1}$) by 'blebbing' out the post-junctional membrane region away from the cell interior[6]. However, studies on the vesicular stomatitis virus (VSV) G-protein, truncated or modified at the C-terminal cytoplasmic tail by means of site-directed mutagenesis, did not show expected increase in the lateral mobility on natural membrane, indicating that the cytoskeleton is not only/always the factor that contributes to the decreased lateral mobility of membrane proteins[7]. In 1988 Wier and Edidin showed that lateral mobility of L^d class I major histocompatibility complex (MHC) antigen is largely constrained by the extent of glycosylation and by the size of the extracellular domains[8]. The protein-density effect on the lateral diffusion was studied by using rhodopsin (an integral membrane protein of rod photoreceptor cells of the retina, which serves as a good model because of its intrinsic fluorescence in the visual range) reconstituted in phospholipid vesicles. The experiments showed that increase in the density of protein decreases the lateral mobility considerably[9]. Thus the inferences that were drawn out of the physical constants obtained for various membrane proteins were found to be of biological relevance. The determinants of the lateral mobility for each protein differ and depend on individual structure–function relationship. This was taken as an evidence to explain why membrane 'resident' proteins such as band-3, acetylcholine receptor, are resident proteins and how 'migrant' proteins such as receptors for transferrin, LDL, asialoglycoprotein can move into coated pits, cluster, experience endocytosis and carry out their function effectively. It also explained how receptor depletion by endocytosis does not affect the rate of further accumulation of R–L complexes in coated pits because of the density effect, until there is no such phenomenon as 'receptor down-regulation' (e.g. a concentration-dependent and time-dependent insulin-induced insulin-receptor down-regulation occurs a short period after insulin treatment because insulin receptor would no longer be able to bind insulin. A recent study shows an insulin-induced proteolytic cleavage of a 61-kDa cytoplasmic tail of β-subunit of the receptor[10]. Ligand-induced receptor-down-regulation also occurs because of the depletion of receptors on the cell membrane as a result of degradation of receptors upon endocytosis).

A critical question that arose at this juncture was whether binding of ligand induces receptors to encounter coated pits or whether receptors spontaneously localize in these pits and are occupied by the ligand. Are receptors like elevators which move only when passengers (ligands) push the button or are they like escalators which move continuously whether passengers (ligands) are aboard? The results of the experiments which were oriented towards these questions came out in favour of escalator model. In the absence of the ligand, the LDL-receptors moved into coated pits where they were visualized by the addition of appropriately tagged ligands or antibodies[11]. Continuous internalization of receptors was also suggested by the observation that the LDL-receptors were trapped inside the cell within 15 min of addition of monensin or chloroquine (which elevates the pH of the endosome, details of which are discussed later) even in the absence of the ligand[11]. Similar ligand independent receptor encounters with coated pits have been reported for the receptors for α-2 macroglobulin and asialoglycoprotein[12]. A calculation made out of diffusion constants predicted that in a 2% area of the total cell surface covered with coated pits, each receptor would encounter a coated pit every 4 to 5 sec (ref. 13).

Components of coated pits and their role in receptor trapping

Whereas studies on the lateral diffusion of membrane proteins gave conclusive evidence as to how R–L complexes could encounter coated pits, it is not clear how they are clustered and trapped in the coated pits, a prerequisite for receptor-mediated endocytosis. Although a body of information implicated the components of coated pits in receptor trapping, studies related to the molecular mechanism behind selectivity and specificity have provided more questions than answers.

Coated pits on the cell membrane are visualized by electron microscopic studies where they are morphologically distinct because of a thick cytoplasmic bordering formed by specialized proteins with a membrane invagination, having a concave curvature facing the cytoplasmic side[14] (see Figures 1 and 2). A surge of rigorous research in the last decade on a host of proteins that renders the structural framework for coated pits revealed that there exist protein structural units of two major kinds: self-associable clathrin and a group of proteins variously termed as assembly proteins, adaptors, associated proteins, accessory proteins or simply as APs (Pearse[15], who coined the term 'clathrin' in 1975, favours the term 'adaptors' due to their 'adaptor' role in receptor trapping as discussed later). These proteins have been isolated at various levels starting from yeast to man and a considerable degree of homology in their primary structure has been established by genomic studies. The functional clathrin unit present in cells is a trimeric complex consisting of three heavy chains and three light chains of two different species; LC_a (~32 kDa) and LC_b (~34 kDa). It is a symmetric structure with three 45–50 nm legs joined at a common hub. Because of this anatomy visualized in electron micrographs, clathrin functional unit is often termed as 'triskelion'[16] (see Figure 3,*a*). In a low ionic strength and low pH condition the triskelions self assemble into 'cages' (a 'cage' is a polyhedral structure made up only of clathrin heavy and light chains or heavy chain alone as opposed to a 'coat' which is a proportionate mixture of clathrin and adaptor proteins) (Figure 3,*b*). The *in vitro* assembly of clathrin has a critical concentration less than 50 $\mu g\ ml^{-1}$ (ref. 16). The cages once formed constitute a heterogeneous population ranging in diameter from 60 nm to 120 nm. Adaptor-protein oligomer, on the other hand, is composed of 16-, 50- and 100-kDa polypeptides. Although there is a general agreement that there are two 100 kDa polypeptides in each complex, the exact stoichiometries of the 50-kDa and 16-kDa polypeptides remain unresolved[16]. Two species of adaptor protein oligomeric complexes have been recognized on the basis of their subcellular localization; HA_{II} which is found exclusively on plasma membrane-coated pits while HA_I is localized to the golgi region

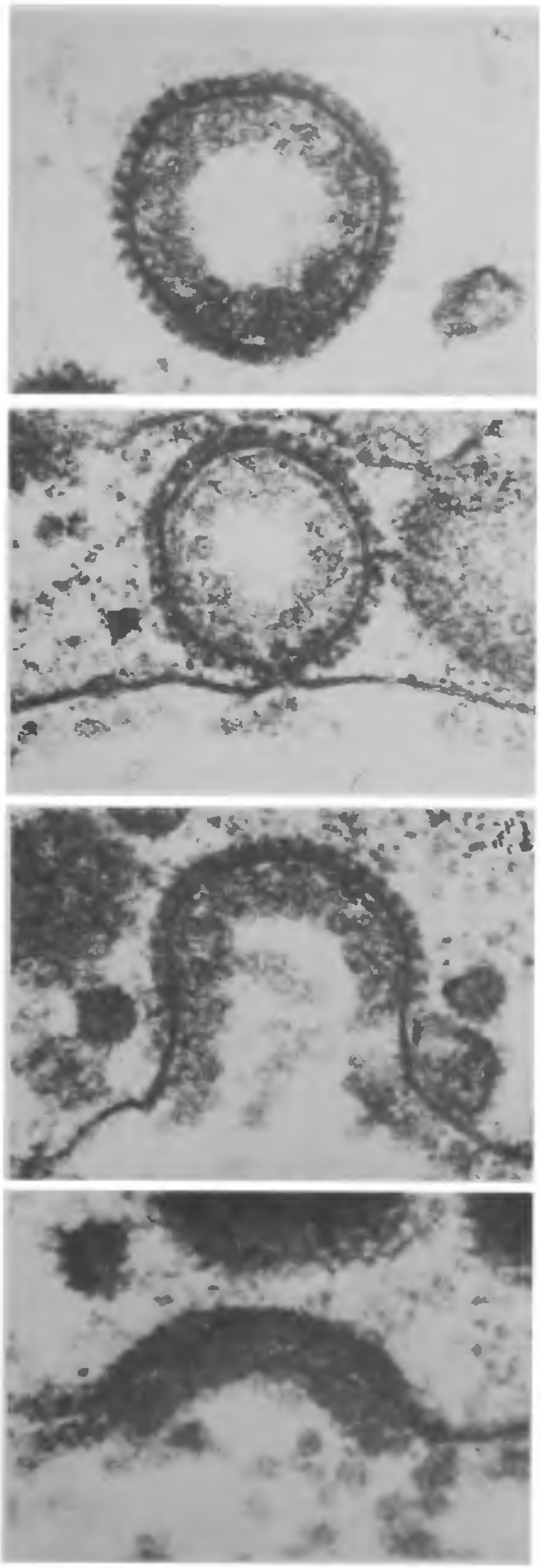

Figure 2. Different stages of formation of coated vesicles from coated pits in the developing oocyte. Oocytes have been a good source for study of coated pits ever since the coated pits were first observed on mosquito oocyte by Roth and Porter in 1964. Almost 60% of the oocyte cell surface is invaginated with coated pits as opposed to a moderate 2% on a fibroblast or a typical tissue culture cell. A single oocyte can ingest 3–5g of yolk proteins in one day with an endocytosis rate sufficient to internalize the equivalent of the total plasma membrane about every 30 min[15]. These electron micrographs taken by Perry and Gilbert[14] show a schematic series in the formation of yolk containing coated vesicles from coated pits of hen oocyte. (Courtesy: The Company of Biologists Ltd., Cambridge, UK)

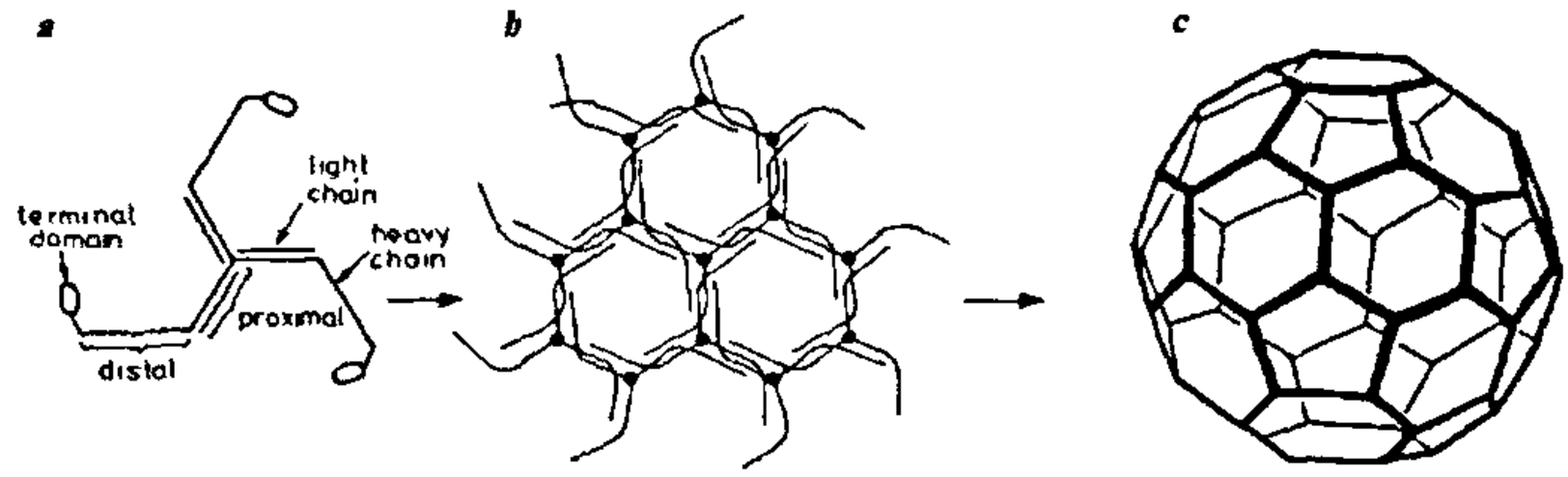

Figure 3. *a*, Schematic drawing showing the modular structure of clathrin triskelion. *b*, Packing diagram showing how triskelions form a hexagonal lattice. *c*, Pictorial representation of clathrin 'cage' structure formed *in vitro*. (Adapted from ref. 14.)

(the nomenclature comes from their elution profile on hydroxy apatite column chromatography)[17].

How do coat proteins assemble into coated pits?

A question of central importance of the coat proteins is their *in vivo* functional assembly and disassembly on the plasma membrane (Figure 1). Although there are a few reports about the preferential localization of coated pits, viz. in microvilli of intestinal epithelial cells, tips of deep plasma membrane invaginations or at the site of granule fusion, the coated pits are generally seen evenly distributed on many cell types[17]. Whether initiation of growth of coated pits requires the presence of specific receptors on the cell membrane has so far eluded an answer. Several lines of evidence suggests that adaptor proteins form a direct contact with the membrane while clathrin forms the outer polyhedral cage, making flexible contacts via its terminal domains with the inner layer of adaptor proteins: first, the clathrin can be released from the coated vesicles *in vitro* under conditions that do not release the adaptor proteins. Second, the adaptor proteins have been demonstrated in three-dimensional reconstitutions of clathrin-coated vesicles in vitreous ice to lie between the clathrin shell and the receptor containing vesicle membrane[17]. Thus it has been suggested that adaptor proteins associate with membrane receptors by a hitherto unknown process, induce curvature in the membrane and nucleate clathrin assembly. Since it is clear from *in vitro* assembly experiments that clathrin naturally forms closed shells (cages), its binding is expected to stabilize convex curvature of the membrane into the cytoplasm. What decides the size of the coated pits has also been addressed by several workers. It has recently been reported that the ratio of the adaptor proteins to clathrin influences coat diameter, smaller structures being formed in the presence of increasing amount of adaptor proteins[18]. This finding is also supported by the fact that cytoplasmic acidification of the intact cell, in which condition the adaptor proteins are expected to undergo high affinity association, causes increase in curvature of the pre-existing coated pits[19].

Adaptor–receptor interaction: involvement of 'tyrosine signal'

Seemingly two independent processes, the adaptor protein binding to membrane, the primary step in the process of formation of coated pits, and receptor trapping in coated pits seem to pose 'the-goose-and-the-egg' puzzle. Explanation as to whether adaptor proteins binding to membrane is receptor-dependent, or the receptor trapping occurs only upon coated pits are fully formed, still awaits substantiation. The fact that the adaptor proteins are retained to a substantial extent on coated vesicles whose clathrin has been removed by mild chemical treatments has indicated that they are not simply trapped within the clathrin lattice but are likely to have direct interactions with binding sites on the membrane[20]. Although no 'one' receptor which could singularly account for the amount of adaptors bound at the coated pits is reported so far, there are reports of high affinity competable binding sites on the brain cell membrane[21]. In this regard, a thorough search for a consensus signal on the cytoplasmic tails of a variety of 'migrant' membrane proteins in terms of amino-acid sequence with potential binding site(s) for coat protein was made. To one's dismay, no two receptor species are found to have any consensus stretch at the cytoplasmic end[22]. Receptors differ greatly in their overall sizes, in the sizes of their cytoplasmic tail and in their orientation in the bilayer. While LDL-receptor, transferrin receptor and asialoglycoprotein receptor, orient their N-terminal end towards the cytoplasm, receptors for FC, EGF, and VSV G-proteins, have their C-terminal end 'hung' in the cell interior[22]. Phosphorylation as a cause for adaptor–receptor interaction on the membrane, an assumption born out of the fact that cytoplasmic domains of many receptors contain potential serine-phosphorylation sites, was analysed in transferrin receptors by site-directed

mutagenesis[23]. The results of such an experiment revealed that cytoplasmic tail is essential for endocytosis of transferrin receptor but not its phosphorylation sites. In the mid-1985, Pearse[24], for the first time, showed that purified mannose-6-phosphate/insulin-like growth factor II (M6PR/IGFII) receptor could form aggregation with adaptor proteins. Later in the same year, it was shown that the LDL and poly Ig receptors with truncated cytoplasmic tail were defective in internalization[22,25]. However, the first clue to the identification of a signal in the cytoplasmic tail for binding of receptors to coat proteins came only from the JD mutant of natural origin (in familial hypercholesterolemic patients), where the amino-acid substitution from tyrosine to cystein in the cytoplasmic domain of LDL-receptors impedes trapping of receptor–ligand complex in coated pits[26]. In the subsequent years, more confirmative evidence for the involvement of tyrosine in receptor–coat protein interaction was made available. In 1988 Pearse[27] showed that adaptor proteins bind to LDL-receptor-tail affinity matrix and that the bound adaptor proteins could selectively be released from the matrix by tyrosine-containing peptide corresponding to the cytoplasmic domain of mutant influenza HA protein (which cluster in coated pits); but the wild-type peptide lacking tyrosine (whose parent molecule is excluded from coated pits) did not compete for binding[27]. A fusion protein containing the cytoplasmic tail of the M6PR/IGF-II receptor binds adaptor proteins, but mutation of the two tyrosins in this tail diminishes the binding of adaptor proteins[28]. Furthermore, in the late-1990, it was shown that a 10-amino-acid stretch of the cytoplasmic tail of transferrin receptor, which contains the sole tyrosine, is essential for endocytosis[29]. A mutant receptor containing glycine instead of tyrosine in that region fails to be internalized efficiently[29]. Thus, there are compelling biological evidences accumulating that 'tyrosine signal' is essential for receptor trapping in coated pits prior to endocytosis. This could also be taken as an evidence to explain why some receptors, viz. influenza HA protein (lacks tyrosine in the 10-amino-acid-long cytoplasmic tail) and Thy-1 (a lipo-protein whose protein part is attached to a glycosyl phosphatidyl inositol lipid molecule present on thymocyte and neuronal cell membranes, and hence lack the cytoplasmic domain), are excluded from being accumulated in coated pits despite their relatively high lateral mobility. Attractive as it looks, 'tyrosine signal' may provide more definitive answers to the physicochemical basis for formation of coated pits and receptor trapping in the years to come.

Coat-protein disassembly

An equally important but opposite process is the uncoating of the coat proteins after vesicle formation which is believed to be essential for the subsequent vesicle fusion and transport process (see Figure 1). Except in the garland cells of *Drosophila* shibire mutant, where coated pits appear to be trapped and are apparently unable to pinch off into vesicle[30], there is no direct evidence of a specific event involving membrane fusion required for the final pinching-off of a coated vesicle (see Figure 2). It is often presumed that pinching-off of coated vesicles is just a physical phenomenon resulting from aggregation of R–L complexes with the concomitant change in the physical status of the membrane pit. The life of an endocytic-coated pit from initiation to pinching-off lasts between a few minutes (based on the uptake of LDL-receptor in fibroblasts)[31] and about a minute (based on the uptake of semliki forest virus in BHK cells)[32]. In a single cell, approximately 1500–3000 coated pits will be transformed into coated vesicles per minute, during the steady-state uptake of simliki forest virus[32]. Uncoating of coated vesicles occurs immediately after the formation of coated vesicles. An ATP-dependent clathrin cage uncoating activity was identified in 1982, and a soluble 70 kDa uncoating ATPase was subsequently purified to homogeneity by exploiting its tight binding to ATP-agarose[33,34]. Further studies by immunological cross reactivity, peptide mapping and two-dimensional gel electrophoresis established that the uncoating ATPase is a member of the multigene heat shock family of proteins of molecular mass 70 to 78 kDa, a highly conserved group of proteins with high affinity ATP-binding sites, whose overall homology approaches 50% from bacteria to mammalian sources[16]. These proteins, fashionably termed as 'molecular chaperones', are probably one of the most celebrated proteins of the last decade because of their 'assistance' in *in vivo* protein-folding process. Although an involvement of one of such ATPases in the uncoating process is beyond any dispute, the dilemma remains; why in the cell, do uncoating ATPases strip off coat from the vesicles but not from the coated pits? or why do coat proteins not rebind to receptors on uncoated vesicles but return to bind only to those on the parent membrane?

Endosome acidification: a principal determinant of R-L dissociation

The different steps involved in the endosomal trafficking of receptor and ligand have been defined by precise kinetic and biochemical terms at least for a few transport types[1,2]. The rate of binding of transferrin, an iron-carrying protein, to the receptor on the cell surface and the rate of subsequent internalization in endosomes assume pseudo-first order process with half time of 3 min and 2.3 min respectively at 37°C (ref. 1). Asialoglycoproteins binding to receptor, on the other

hand, has a half time of 6 min and that for internalization is 1.5 min in hepatocytes[2]. These rates appear to be independent of receptor occupancy. The endosomes carrying so internalized receptor–ligand complex are also called as receptosomes[13]. The vast heterogeneity in size, shape and contents of the endosomes have made it difficult to define precisely the early phase and the late phase of these structures, and differentiate them from other organelles, viz. lysosomes. Nevertheless, based on the presence of endocytosed ligands detected by electron microscopic studies, it was determined that endosomes account for 1% of the cellular volume but comprise more than 25% of the total cellular membrane[35]. These endosomes are seen concentrated at the peripheral region of the cytoplasm. Although differential transport of different types of receptor and ligand in these vesicles seem to rely on individual structure–function relationship of the receptor and ligand (discussed below), there are some common criteria that are met with by most of these transport types. An early observation that half lives of most of the receptors are much longer than that of the ligand, has led to the postulate that the receptor recyles back to the cell surface, maintaining a stringent cell-economy, and the ligand goes to a place where it is destined to. This has emphasized two points: (i) R–L complexes should dissociate upon their internalization, and (ii) seggregation of receptor and ligand must occur in a cytoplasmic compartment with high fidelity. Organelle acidification seemed to be a plausible way in which receptor–ligand dissociation could occur. The first indication that cells have intracellular low pH compartments occurred when Metchnikoff, in 1893, observed that, litmus paper phagocytosed by certain protozoa changed colour from blue to red[36]. Ever since, acidic compartments, particularly in unicellular organisms and lysosomes of higher organisms, have been extensively studied using vital techniques[36]. However, the first hint that the lumen of endosome is also acidic came only in 1980, when Helenius *et al.*[37] observed that the fusion of enveloped viruses with the endosome membrane, an essential step in the viral infection which needs a low pH environment, occurred shortly after their internalization, much before the vesicles could reach the lysosome — the degradative compartment. A new development took place in 1982, when Tycko and Maxfield[38] demonstrated that, 15 min after the endocytosis, the fluorescein-labelled α-2-macroglobulins encounter an acidic environment as indicated by the change in the emission spectrum. This interval was felt to be too short for the ligand to have reached the lysosomes, a conclusion that was confirmed by histochemical electron microscopy. Based on the premise that weak bases, such as chloroquine, accumulate in acidic intracellular compartments, weak bases have been found that are both retained at sites of accumulation upon aldehyde fixation in histochemical sections and detectable by electron and light microscopic immunocytochemistry. One of such probes, 3-[2,4,-dinitroanilino]-3'-amino-*N*-methyldipropylamine (DAMP), developed by Anderson[39] and co-workers not only accumulated in acidic compartments but also enabled one to 'see' the acidic compartments by fluorescently labelled or gold-labelled monoclonal antibodies that recognized dinitrophenyl group, in cytochemical studies. Based on the extent of accumulation of weak bases, as could be quantified either by counting the number of gold-particles or by the intensity of fluorescence, depending on the probe used in the technique, almost precise pH values for endosomes (~5.6), lysosomes (~4.6) and trans-golgi compartment of golgi apparatus (~6.5) have been asigned[39].

How do endosomes maintain acid pH?

To date, several proton ATPases have been characterized which are involved in maintaining the pH of different organelles and the cytosol as a whole (see Figure 4). Three general categories of proton ATPases in eukaryotic cells: ATPases of the mitochondrial-bacterial F_1F_0 class, the plasma membrane proton ATPases and vacuolar ATPase have been classified based on their sensitivities to a variety of inhibitory agents as well as their general functional characteristics[36]. Origin of vacuolar ATPases is still not clear. While coated vesicle is also found to contain proton ATPases[36], it is assumed that vacuolar proton ATPases are originally present on the plasma membrane and transferred to coated vesicles, where, because of the increased surface to volume ratio, maintain low luminal pH. The mechanism behind the maintenance of number of proton pumps in each endosome and regulation of pH

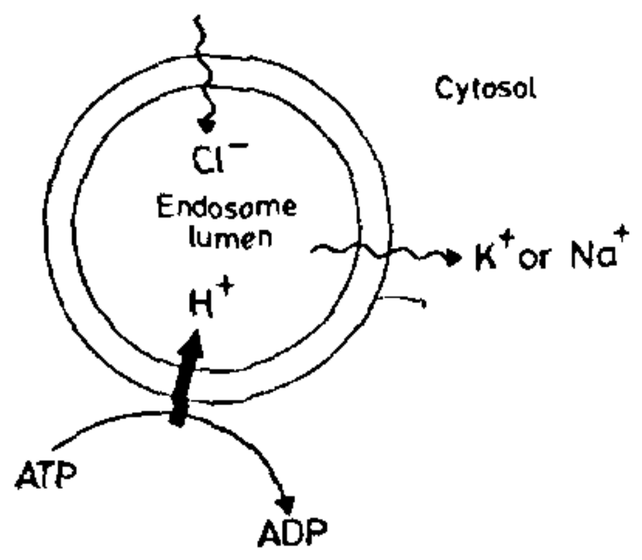

Figure 4. Pictorial representation of the mechanism of ATP-dependent acidification of endocytic organelles. A vacuolar proton ATPase catalyses the electrogenic translocation of protons into the vacuole, creating both a positive internal membrane potential and a low internal pH. The membrane potential is dissipated by passive influx of permeant cytosolic anions (Cl^-) or efflux of internal cations (K^+ or Na^+). (Adapted from ref. 35.)

in the vesicle lumen remain as yet unknown. Some striking observations were made as for the rate of endosome acidification by using biological approach. In 1986 Kielian *et al.*[40] showed that entry of virus could be a potential probe for measuring the rate of acidification of endosomes. One of the well-studied viruses, semliki forest virus has spike glycoproteins, which, upon the entry of the virus in the low pH endosome, mediate virus envelope fusion with the endosome membrane, so that the virus genome enters the cytosol for further propagation. A mutant virus has a fusion pH threshold of 5.2 as opposed to 6.2 of that of the wild type. The rates of entry of both the wild type and mutant viruses were found to be the same ($t_{1/2} = 10$ min). Fusion event was detected by the extent of release of radioactive RNA into the cell cytosol. The half-time for fusion of wild type virus was 15 min after its entry, whereas that of mutant was 35 min, both of which reflected the rates at which they reached their respective fusion pH threshold. Interestingly, the rate of fusion of the mutant virus was 'asynchronous', indicating that acidification in some endosomes occurred faster and that, there was no synchrony in the rate of acidification of newly formed different endosomes. This is believed to be a cause of difference in the number of proton pumps in each endosome and the endosome size[40].

R–L complexes typically dissociate at acid pH

The discovery that endosome is also an acidic organelle is considered to be an important development in understanding the mechanism of receptor recycling. Receptors are often found to bind their ligands with high or intermediate affinity at neutral pH (the pH of the extracullar medium) and only weekly in mildly acidic pH (the pH of the endosome). The R–L complexes formed on the plasma membrane therefore dissociate when they reach the endosome. The molecular basis for the change in binding affinity is not clear. The limited data available suggest that it is primarily the receptor that reacts to the low pH. Several of the receptors, viz. asialoglycoprotein receptors, mannose receptors and the mannose 6-phosphate receptors have 'lectin' activity and mediate uptake of a large variety of glycosylated ligands, including 'neoglycoproteins' with artificially attached carbohydrate side chains[41]. Dipaola and Maxfield[42] found a large-scale conformational change in asialoglycoprotein receptor upon lowering the pH from 7.0 to 5.6 by monitoring change in the limited proteolytic products and extent of radioiodination. It is assumed that asialoglycoprotein receptors (pI ~ 4.7) will have decrease in net charge upon exposure to low pH, which might allow the protein to fold into a more compact form. In 1987 Davis *et al.*[43] showed that a domain in the LDL receptor containing 3 cystein-rich 'growth factor repeats' is responsible for release of LDL from the receptor in the acidic endosome. When this domain is deleted, the receptor no longer releases its ligand at acid pH, it is no longer recycled efficiently and is rapidly degraded after ligand binding. The prime example where the ligand is sensitive to acid pH, is the iron-transporting protein transferrin. Diferric transferrin binds to the receptor on the cell surface, the R–L complex rapidly undergoes endosomal transport, releases iron in the acidic compartment and recycles back to the cell surface. This is an elegant economical pathway which depends on carefully adjusted pH dependences between the transferrin and the receptor on one hand, and between the iron atom and transferrin molecule on the other. While transferrin has quite a high affinity for its receptor at neutral pH (2.1×10^{-9} M), apotransferrin has high affinity at acidic pH (2.1×10^{-8} M at pH 4.8), but relatively low affinity at physiological pH (4.8×10^{-8} M) (ref. 44). The iron which is extremely tightly bound to transferrin at neutral pH, on the other hand, dissociates easily at mildly acidic pH. These facts, in conjucture with the low endosomal pH, suffice to explain, why transferrin with two iron atoms binds to the cell surface receptor, goes to endosomal low pH compartment where it releases iron, but avoids being released and moves back to the cell surface in its apoform, where, at physiological pH, it dissociates from the receptor. *There is one more class of R–L complexes* where neither the receptor nor the ligand is affected by low pH. The best examples are, choriogonadotropin and immunoglobulins where the R–L complexes are either degraded in lysosomes or transported across epithelial cells[45,46].

CURL

The year 1983 is marked in cell biology for the discovery of an intracellular compartment designated as CURL (compartment of uncoupling of receptor and ligand) where receptor and ligand seggregate (see Figure 1). Geuze *et al.*[47] used double-labelling immuno-electron microscopy on ultrathin sections of rat liver to identify the site at which the asialoglycoprotein receptor dissociates from its ligand. Upon endocytosis ligand initially encounters endosomes with relatively small vesicular volume. Thereafter the vesicle volume increases and produces a tubular protrusion. About three-fourths of the total membrane appears to be in the tubular portion, while about three-fourths of the volume is within the vesicular portion. Computer-assisted three-dimensional reconstructions of endosomes from serial thin sections of BHK-21 cells, revealed populations of tubular vesicular structure with an average of six tubular arms up to 4 μm long[48]. The vesicular portions have diameters of 0.1 to 0.5 μm, while the diameter of the tubular portions is approxi-

mately 0.05 μm. Seggregation of the ligand and receptor occurred in a population of such tubular vesicles, the CURLs. The receptors were attached to the membrane of the tubular extension whereas the ligands were free within the lumen of the vesicle. The interpretation that followed these observations is that the tubular structure containing exclusively the receptors gets detached from the vesicular portion that contains exclusively the ligand, and returns back to the cell surface (see Figure 1). Although this seems to be an elegant way in which receptors recycle back to the cell surface leaving ligands behind, so far there is no explanation as to why only receptors diffuse into the tubular extension but not ligands. Linderman and Lauffenburger[49] gave a rigorous theoretical treatment to the problems related to the diffusion of receptor and ligand in CURLs. Result of such an effort was found to be paradoxical to the experimental observation. They obtained diffusion coefficient for ligands which was two to three orders of magnitude higher than that obtained for receptors. However, the authors contend that the receptor experiences the advantage of lower dimensionality in its search. The time a receptor takes to find a tubular entrance will depend on the vesicle radius, the appropriate diffusion coefficient, the number of tubules connecting the vesicles, and the size of a tubular entrance[50]. In addition, these results have also led to the postulate that receptors are probably trapped in the tubular extension upon their diffusion possibly because of the presence of coat proteins or similar structures, or because of receptor self-aggregation induced by acid pH. The fact that a small fraction of ligands also recycles back to the cell surface along with receptors, is taken as an evidence that not only receptors but also ligands diffuse into the tubule, but to a lesser extent[1].

Vectorial transport: a perennial problem

From the discussion that precedes, it is clear that, at one point or the other, all the transport types show a certain degree of directionality. In many receptor–ligand systems, the receptor recycles back to the cell surface and the ligand enters a degradative compartment—the lysosome. Lipoproteins of LDL are subjected to proteolytic cleavage, while cholesterol is further 'processed' for the biosynthesis of membrane. A similar vectorial-transport mechanism exists for the clearance of asialoglycoprotein from the serum, where the asialoglycoprotein receptors recycle back and the ligand will be transported to lysosome for degradation. The directionality in the movement of vesicles carrying various receptor and ligand is expressed to its maximum in polarized cells such as epithelial cells and neuronal cells, where all the sides of the plasma membrane are not equal. The mucosal epithelial cells throughout the body carry out transport of immunoglobulin in a variety of ways across the cell (transcytosis) and release them either in the intercellular space or in such lumens as bile canaliculi. This is essentially a defence stretegy against any pathogen. In neonatal rat, maternal IgG is transported across the intestinal epithelium; the IgG first binds to Fc receptor on the brush border membranes of the epithelial cells that line the duodenum and proximal jujunum[51]. The binding of IgG to receptors is pH-dependent; IgG binds at pH 6.0–6.5 but not at pH 7.4. The IgG taken up by the cell is transported vectorially to the lateral surface of the cell and is released into the intracellular space. Another example is the vectorial transcytosis of polymeric IgA across the hepatocytes into the bile canaliculus[46]. In some animal species, viz. rat, chicken and rabbit, this process occurs through a specific receptors called secretory component (SC) receptor. They number approximately 200,000 on each hepatocyte[46]. Polymeric IgA binds to these receptors with an affinity of 10^{-8} M, gets internalized into endosomes. During the vectorial transport of vesicles to the basolateral membrane, the secretory component gets covalently bound to the polymeric IgA and separate from the membrane spanning part. Unlike many other receptor types, these receptors, once endocytosed will not be utilized again for the transcytosis and hence the name 'sacrificial' receptors.

Yet another example of vectorial transport type, but incompressible in concrete terms because of intricate, yet ununderstood mechanisms involved in the process is the antigen processing by antigen processing cells (APCs)[52]. Circulating antigens are taken up by macrophages or by B-lymphocytes, processed in the intracellular vesicles into peptides. These peptides are recognized by two classes of glycoprotein antigen-presenting molecules; class I MHC and class II MHC. As a consequence of different pathways of intracellular traffic, class I molecules bind peptides derived from endogenously synthesized proteins, whereas class II molecules bind peptides derived from exogenous proteins that have entered the cell by endocytosis[52]. MHCs target the peptides to plasma membrane where the complex of MHC plus peptide is presented to receptors of circulating T-lymphocytes during the process of production of antibody. How do these MHCs avoid lysosomal degradation? How are peptides of precise lengths formed which are not observed in other transport types? How are these peptides transported to the cell surface of APCs despite the low pH environment of endosomes? These are just sample questions of general interest.

Although there is no concrete mechanism divulged impeccably for any one of these vectorial transport types, circumstantial evidence suggests that the pH, the receptor-ligand affinity and the ligand 'valency' are the

prime factors that decide the fate of the receptor and the ligand. When endosome acidification is inhibited by the addition of acidotropic agents or carboxylic ionophores, intracellular receptor-ligand dissociation is fully or partially blocked and the complex is accumulated in the highly swollen endosomes[53]. The constitutively internalized receptors in the absence of ligand also accumulate in the vacuole in such circumstances[10]. LDL-receptor without the 'growth factor repeat' domain which is essential for receptor-ligand dissociation in low pH compartment, is rapidly degraded in lysosome[43]. That the outcome of sorting process for one particular receptor system may also depend upon ligand 'valency' is shown by Mellman[54,55] and coworkers. When macrophage Fc receptors are bound by monovalent Fab fragments of an anti-receptor antibody, the receptors are recycled efficiently and much of the ligand is exocytosed. However, binding of the same receptor with multivalent ligands results in decrease in the receptor half life from 15 to 5 h as more receptors are routed to the degradative pathway. Similar observations were made for anti-receptor antibodies to LDL-receptor and transferrin-receptor[56,57]. Involvement of microtubule-based 'kinesin' motors is reported in the interorganellar vesicular transport at least in neuronal cells where the vesicle transport experiences the longest length ever possible in any cell type known so far[58]. The nerve transmitters synthesized at one end will be transported to the presynaptic area along the exon. The microtubule-based kinesin motors are operated by mechanochemical coupling in which force-generating proteins provide movement along microtubule arrays similar to the system of myosin-based movement on actin. The video-enhanced technology has demonstrated the movement of vesicles of approximately 30 nm in diameter along the exon at rates up to $5\,\mu$ sec^{-1}. Whether a similar mechanism exists for the endosomal transport is yet to be discovered.

Conclusions

A 'round trip itinerary' of a receptor occurs time and time again involving molecular events discussed hitherto. Asialoglycoprotein receptors and transferrin receptors take approximately 15 min for one such trip[1,2]. In isolated Hep G_2 cells, uptake of transferrin occurs for at least 4 h, at a steady-state rate of 9500 protein molecules/cell/min, during which period each receptor is calculated to be utilized about 15 times independent of new receptor synthesis[1]. Each LDL receptor makes one round trip every 12 min and 150 round trips in its 30 h life span[11]. By circulating selected proteins and lipids in coated pits and vesicles, cells ingest nutrients, degrade hormones, and maintain the protein and lipid compositions of their organelle membrane. Recent advancement in our knowledge of these processes includes the mechanism in which NEM-sensitive fusion proteins carry out fusion of vesicles during endosomal transport of receptor and ligand[59]. An interesting, but a peculiar phenomenon that has been brought to light in recent years is the ability of some cells, often termed as MDR-cells (MDR for multi drug resistance), to maintain low concentration of drug in the cell interior, particularly the alkaloids or antibiotics of plant and fungal origin, by a specialized mechanism involving a 170-kDa protein called P-glycoprotein[60]. This pH-sensitive efflux of drugs is also believed to utilize endosomal transport machinery, the nature of which is not clearly understood. Another important factor that is gaining much consideration only in recent years is the role of lipids in vesicle trafficking. Simons and van Meer[61], who demonstrated a polarized delivery of lipids by vesicular transport and also a substantial difference in the composition of lipids of the apical and basolateral membranes of intestinal epithelial cells, strongly believe that the intracellular protein trafficking cannot be understood apart from lipid trafficking. Research in this prime area is progressing at a strident pace and the present decade will certainly usher a deeper understanding of these molecular events.

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REVIEW ARTICLE

Diabetes and avian resistance

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In comparison to other vertebrates, the birds are more resistant towards experimental manipulations which cause onset of diabetes mellitus. In contrast to mammals spontaneous diabetes has been reported in some parakeets only. In this paper we discuss various plausible factors behind this refractoriness. Significance of glucagon/insulin ratio, high avian somatostatin and avian polypeptide values in preventing diabetes have been emphasized.

THE advent of comparative approach to endocrine problems has sharpened the focus of many investigators on the usefulness of projecting the bird into the forefront of diabetes mellitus-related studies. Diabetes mellitus is a metabolic disorder characterized by clinical symptoms resulting from real or apparent insufficient pancreatic secretion of insulin or overabundance of some insulin-inhibiting factors.

Spontaneous diabetes mellitus

In addition to the prevalence of spontaneous diabetes in humans, it has also been observed in domestic and captive animals like horse, cattle, pig, sheep, dog, cat and monkey[1]. Among aves, spontaneous diabetes mellitus as a clinical entity to our knowledge has only been reported in some parakeets (*Melopsittacus undulatus*)[2]. The parakeets showed polydipsia, polyurea, weight loss, high-urine glucose and elevated blood