THE EARLY DAYS OF PINOCYTOSIS

by

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1. INTRODUCTION

The earliest suggestion of “cell drinking” appears to be the medically orientated theory put forward by the American physician MELTZER in 1904 (51). This hypothesis, for which there was no experimental evidence, was clearly influenced by METCHNIKOFF'S (52) description and definition of phagocytosis in 1883. If cells can eat, why should they not also drink? MELTZER'S theory sought to explain the formation of oedema by the transport of fluid through cells, which “sipped” fluid from the surrounding medium, a process which this author called “potocytosis”. Apart from an observation by EDWARDS (27) in 1925 on food cup formation induced by neutral inorganic salt solutions in amoebae, there were no further published data or theories on fluid uptake until 1930. In this year, a short editorial in the *Journal of the American Medical Association* by MAINWARING (48) under the title “Hydrophagocytosis” brought to the attention of physicians the cine films of the movements of rat macrophages in culture which LEWIS had demonstrated at meetings in the United States. MAINWARING pointed out that “the Lewis phenomenon”, during which the macrophages captured and swallowed plasma from the surrounding medium, could be of importance in cellular nutrition, and in “mechanical filtration of purification of body fluids”, as well as “disposal of soluble antigens and .. synthesis of specific antibodies”. Then in 1931 LEWIS (46) published

photographs and descriptions of this process, showing that during active movements at the cell surface, droplets of the fluid medium were engulfed; these droplets were at first of irregular shape, but became spherical as they passed into the interior of the cell, sometimes fusing with other droplets, and rapidly decreasing in size. It was in this classical paper that the term pinocytosis first appeared, at the suggestion of Lewis' colleague, DAVID M. ROBINSON; the designation was derived from the Greek, *πινειν* to drink, and *κυτοζ* cell. Later LEWIS (47) observed the same phenomenon in cultured malignant cells. Thus the term pinocytosis appeared in the literature 50 years after METCHNIKOFF had described and defined phagocytosis.

The observations of MAST and DOYLE (49) in 1934 on the uptake of fluid by freshwater amoebae, a process for which these authors also used Lewis' term pinocytosis, differed in two respects from LEWIS' descriptions of the process in macrophages; in the macrophages droplets were engulfed directly at the cell surface, while the amoebae formed channels on rather immobile pseudopodia, at the base of which vacuoles were pinched off from the channels. The other difference between pinocytosis in these two cell types was that while the macrophages continually formed droplets from the nutrient medium, the amoebae required a “stimulus”, in the form of diluted sea water or egg albumin, before pinocytosis could take place: it should be noted here that while the macrophage medium was a nu-

Figure 1. a and b. Pinocytosis in rat omental macrophages. a. Redrawn from MAST (46), the first paper on pinocytosis. b. Phase contrast photograph (CHAPMAN-ANDRESEN). c and d. Pinocytosis in fresh water amoebae. c. Drawing from MAST and DOYLE (49), the first paper on this subject. d. Phase contrast photograph (CHAPMAN-ANDRESEN).

trient medium, the amoeba medium, which was a low concentration of inorganic salts, could not be considered as nutrient. Higher concentrations of salts and of albumin, a protein, could however be nutritional importance, hence the early emphasis on this aspect of the significance of pinocytosis. These early observations on macrophages and amoebae were all made on living cells without the aid of phase contrast or other accessory optics, but they give clear and full

details of the morphology of the process (see Figure 1).

Meanwhile, since METCHNIKOFF'S first studies and his further exploration of phagocytosis through many animal groups from amoeba to man, considerable data had been accumulated from in vivo and in vitro observations on a wide variety of cells, which phagocytosed living or dead bacteria, organic or inorganic particles of different types. The significance of phagocy-

toxis-enhancing factors from normal and sensitized serum was realized, and the influence of many factors, such as temperature, salts, pH and hormones was studied. Reviews such as those of MUDD et al. (53) and of BERRY and SPIES (1) summarize the extensive research in this field up to 1949. It can thus be seen that phagocytosis had early proved to be a subject of interest to medical physiologists, and was soon attributed a role in certain diseases and in normal body repair functions. However, the concept of pinocytosis, during the first two decades after its first description, was either ignored, rejected as pathological in mammalian cells e.g. by BESSIS (2), or considered as a normal cellular process only in cultured cells and in Protozoa.

2. STUDIES ON PINOCYTOSIS AT CARLSBERG LABORATORIUM

The above introduction shows briefly the background against which studies on pinocytosis were started at Carlsberg Laboratory, first at the Cytochemical Department, and from 1956 at the Physiological Department under HEINZ HOLTER, who had again spotted an important field in cell biology, which could be developed with the material and techniques available at Carlsberg.

Cultures of the fresh water amoebae *Amoeba proteus* and *Chaos carolinense* (then known as *C. chaos* or *Pelomyxa carolinensis*) had been kept in the laboratory for some years, for biochemical, physiological and cytological studies, often using microtechniques, which permitted various types of measurements to be made on single amoebae; studies with radioactive tracers were also in progress. HEINZ HOLTER, together with a newly arrived guest from U.S.A., JOHN M. MARSHALL JR., then commenced studies on pinocytosis in fresh water amoebae. In these first experiments, the uptake of a protein, fluorescein labelled gamma globulin, was explored by measurements of fluorescence in single amoebae. This (43) was the first of a series of studies on different aspects of pinocytosis which issued from HOLTER'S laboratory during the next two decades, the results of his collaboration with guests from Denmark and abroad and with the laboratory's own co-workers.

After confirmation of the need for protein or salt (8, 24) in the medium before pinocytosis could take place, it soon became clear that not all salts or all proteins were equally effective "inducers", as the solutes which were responsible for the initiation of the process were now called. Using a quantitative method for estimating the intensity of pinocytosis, based on the mean number of channels formed under standard conditions, the inducing capacity of a range of water soluble proteins of differing iso-ionic point was explored. The data showed that under conditions where the predominant charge of the protein was positive, pinocytosis was induced (9). In general, non-toxic inorganic salts were effective inducers at approximately neutral pH, although calcium salts were an exception: non-toxic basic dyes were also good inducers.

Further experiments with these different types of inducers confirmed the importance of the interaction between cell surface and inducer for the initiation of pinocytosis; e.g. the presence of fluorescein-labelled protein or basic dyes could be visualised on the cell surface. This first phase of surface binding was independent of temperature (9) but was influenced by conditions in the medium. Changes in the pH of the medium and in its ionic strength, however, influenced different types of inducers in different ways, indicating that surface binding was several types, showing varying degrees of lability. These observations led to the division of inducers into three types, according to the factors which led to the release of the inducer from the cell surface, or in modern terminology "enhance the receptor-ligand dissociation". Inorganic salts, as well as neutral salts of amino acids were effective as inducers only when the active solutes were present in the medium: on transfer to a non-inducing medium pinocytosis ceased immediately. Surface-bound protein was not released on transfer of the amoebae to a non-inducing medium of the same pH as that of the inducing solution, while immediate release occurred if the pH of the non-protein medium differed (19). For basic dyes, the conditions were similar to those shown by protein inducers. Finally, in a small group of compounds, so far comprising the copper phthalocyanin dye alcian blue, and rhuthenium red, the dye, once bound

Figure 2. Diagrams of different, microscopically visible modes of ingestion in amoebae. a. Typical pinocytic channel in salt solution. b. Pinocytic channel in solution of tobacco mosaic virus. c. "Bottle-shaped" cavity in solution of tobacco mosaic virus. d. Cavity formed in optically clear methionine solution. e. Formation of food vacuole containing small ciliate. From CHAPMAN-ANDRESEN and PRESCOTT (24).

to the surface, remained firmly attached despite all possible changes in pH or ionic strength of the medium, that living amoebae could tolerate (9, 10).

The second phase of pinocytosis, the active phase of channel formation, which followed rapidly after surface binding, was dependent of temperature, and sensitive to inhibitors of glycolysis and oxidative phosphorylation (11). During intense pinocytosis active locomotion ceased, and the contractile vacuole ceased to function. The appearance of the channels at the tips of non-locomotory pseudopodia was rapid, and the channels exhibited peristaltic movements as they disappeared into the organelle-containing cytoplasm at their base. The width of the channels depended on the type of inducer, being very narrow, ca. 1 μm , in acid protein solutions, and to 5 μm in some basic dye solutions. In fact a whole spectrum of channels types could be observed, the largest being the bottle-shaped vacuoles formed in virus solutions (24) (see Figure 2).

After these morphological observations, interest returned to quantitative measurements on

the uptake and fate of material ingested by pinocytosis. Further methods available at that time for measuring uptake was the use of radioactive compounds as tracers; rather few were then available compared with the vast range now commercially produced. In the first experiments, glucose, labelled with C-14, was used, as carbohydrates had been shown to be non-inducing (17), and hence might give a measure of the volume of fluid ingested. Non-radioactive albumin was used to induce pinocytosis, and the fate of the ingested material was followed by direct measurements of radio activity and by autoradiography. The glucose was metabolised by the amoebae, thus showing that it penetrated the pinocytic vacuoles, and that in addition to water, significant amounts of solute entered during pinocytosis. In further studies (18) an attempt was made to differentiate between the uptake of fluid and that of surface-bound solute. C-14 glucose was again chosen as the label for fluid uptake, while serum albumin, labelled with I-131 was used to indicate uptake of solute. Comparison of the ratios of isotopes in the inducing solution and within the amoebae after pinocytosis showed that the albumin was concentrated during pinocytosis, and that the entry of fluid was low. Adsorption of the albumin to the filaments of the mucous coat could be seen by electron microscopy; the glycocalyx had become extended to about three times its usual length of ca. 100 nm. The weight of protein entering by pinocytosis could amount to ca. 40% of the dry mass of the amoeba. Although the use of glucose, which is metabolized by the amoebae, as the marker for fluid uptake was by no means ideal, yet these data showed the very large amounts of solute which could enter the cell during pinocytosis. In later experiments using I-131 labelled albumin as inducer, it was shown that the pinocytosed albumin was degraded within the cell, indicating that pinocytosed protein followed the normal digestive path in the amoebae (6). Radioactive tracers were also used to measure pinocytic uptake of inorganic salts (16).

Using a different type of protein marker, the iron core of ferritin, the uptake and fate of this protein was studied, using the modern method of atomic adsorption for measuring the iron

content of the protein, and the old method of *in vivo* centrifugation for following the association of the protein to different types of cell organelles, and for measuring the turn over of lysosomes (15). In addition to the light microscopic studies on living amoebae, fine structural studies were begun; the first electronmicrograph of a pinocytic channel, induced in *Amoeba proteus* by a solution of sodium glutamate, perhaps not very elegant by modern standards, but clear, was published in 1960 (21). Further fine structural studies reflected differences in binding lability of the inducing solutes with the appearance of the mucous coat, or glycocalyx, and the details of the first phases of pinocytosis of alcian blue (29). This useful copper phthalocyanine dye, visible by both light and electron microscopy, was also a marker in studies aiming to relate the path of pinocytosed material through the cell, with that of the path taken by the normal food of the amoeba, living ciliates. Here again, a double labelling technique, with *Stentor polymorphus*, which contains symbiotic green algae, was the marker for normal digestion (phagocytosis) and alcian blue the marker for pinocytosed material, both markers visible by light and electron microscopy (12, 22). By following a number of carefully timed sequences, it was found that the alcian blue labelled secondary lysosomes, which then fused with the *Stentor*-containing food vacuoles, as well as with each other, so that finally both labels were found before egestion in the same vacuoles. Lysosomal enzymes, as indicated by acid phosphatase, were also found in these vacuoles up to egestion (20).

Further studies on pinocytosis of inorganic salts, and the inhibiting action of calcium early found to differ in its inducing capacity (5, 25) refocused attention on the cell surface, on the mechanism of the first phase of induction, on the idea of the glycocalyx as a type of ion-exchange resin, containing weakly acid groups, and on the significance of the permeability of the plasma membrane, with its changes in transmembrane conductance and impedance (4).

In all the studies so far mentioned in this section, fresh water amoebae were used as the experimental animals. However, other organisms and cell types were also included. The uptake of fluorescein-labelled plasma proteins

was explored in a wide range of vertebrate cells (42, 44), and indicated that while cells of the reticulo-endothelial system, of kidney tubules, and of young intestinal epithelium, and also cultured malignant cells and various types of leucocytes ingested the protein, other tissue cells appeared to be inactive in this respect. Other studies included the uptake of fluid droplets from a nutrient medium by the ciliate *Tetrahymena* (23), uptake by plant cells (3), and by cells from mammalian peritoneal exudates (7).

Lectures and reviews by HOLTER (30 - 41) brought the experimental studies of the laboratory, many of which were published in Carlsberg Laboratory's own Journal, to a wider audience.

The excellent working conditions and facilities of the laboratory, the free atmosphere, where one could do "one's own thing", but have access to the support which discussions, suggestions and criticisms from HOLTER gave, all contributed to the progress of these studies, which were only one of the many fields of research in the laboratory.

With HOLTER'S retirement in 1971, and the ensuing changes and reorganization of the Physiological Department, the study of pinocytosis passed from Carlsberg Laboratory. However, some of these studies continue in Copenhagen, at the University's Institute of Cell Biology and Anatomy, where the first observations on vacuole formation in *Tetrahymena* (23) have led to a series of papers by NILSSON (55, 56, 57, 61), on various aspects of uptake, combined with physiological and fine structural studies. Fresh water amoebae are still cultured at the same Institute, at which studies on pinocytosis still proceed (13, 14). These studies at the University of Copenhagen have been made possible by the generous support of the Carlsberg Foundation.

3. CONCLUSION

This account of studies on pinocytosis at Carlsberg does not intend to imply that pinocytosis was not studied, and studied extensively as time passed, in other institutions. However, it is not appropriate here to mention more than a few of the many developments which took place during this period. Following LEWIS' classical

studies on cultured cells, GEY and co-workers (28) and ROSE (58) continued with cinematographic methods and analyses to collect data on different types of normal and malignant vertebrate cells in culture, especially with respect to the inter-relationship between the pinocytic vacuoles and other cellular organelles, and the fate of the vacuoles.

Other interesting studies on amoebae include RUSTADS observations (59) on orientation of basic dyes at the cell surface, during the first, adsorptive phase of induction; competition was found between basic dyes and basic proteins, indicating acidic sites at the surface. Other approaches were those of JOSEFSSON and co-workers (45) who tested the influence of drugs on pinocytosis on inorganic salts, and the interaction between different cations. A series of papers exploring fine structure during pinocytosis was started by the BONN group (62), together with the fate of different types of inducers. Further studies on surface binding of proteins and their fate were made by MARSHALL and co-workers (50, 54), using ferritin and methylated ferritin, two proteins of similar structure, but differing in solubility and iso-ionic point.

From these and other data, it became gradually clear that the distinction between pinocytosis and phagocytosis as originally defined, i.e. between uptake of fluid or of solid material, was tenuous, as in both cases solute and fluid entered the cell simultaneously, and in both cases initiation was dependent on cell surface interactions. Numerous other designations had been given to uptake processes, which have essential similarities with pinocytosis and phagocytosis, these are listed in (9). In 1963, a new designation "endocytosis" was proposed by DE DUVE (26) to cover all uptake processes involving active participation of the plasmamembrane in the formation of vacuoles or channels, which contain components of the extracellular medium, and of which the plasmalemma forms the limiting membrane. This term is now often used in place of pinocytosis, although the latter is still used, especially when the vacuoles formed are large, and the fluid component considerable.

Finally, as an echo from the early days of pinocytosis and its sequels at Carlsberg Laboratory, I was interested to notice, among the

first references in a paper from 1983 "Phagocyte-pathogenic microbe interactions" (59), two reviews on pinocytosis, the first by HOLTER from 1959 (32), the second by a former co-worker (14).

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