INTRACELLULAR TRANSPORT IN THE AMOEBA *CHAOS CHAOS*[1]

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It is often assumed that the transport mechanisms which maintain the internal environment of the cell and provide the materials required for growth and activity are localized directly in the cell membrane. This concept is taken for granted in most discussions of cell physiology and is in fact supported by a considerable body of indirect evidence. Electron microscopists have therefore looked for specialized structural features in the outer membranes of cells which might be equated with the pores, pumps, and shuttling devices inferred from physiological data. The search has not been very rewarding, however, and as a result there has been increased interest in more dynamic concepts of membrane structure and function. These are at two different levels, and have in the past occupied workers in rather different disciplines. On the one hand, there has been discussion regarding molecular fluctuations, statistical pores, and specific carrier cycles—all representing dynamic events at or near the molecular level—which are usually conceived to occur within a larger static framework, "the cell membrane." On the other hand are considerations of membrane uptake and renewal, of dynamic exchanges occurring on a somewhat larger scale, as, for example, in pinocytosis and phagocytosis.

Even though we infer from electron microscopy that membrane movements and uptake must occur in a wide variety of cells, the physiological implications of such activity remain uncertain because of the difficulty of obtaining quantitative information. For reasons which are chiefly technical, the free-living amoebae are the only cells for which we have estimates of the rates of membrane uptake and of the amounts of water and solutes transported under reasonably

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defined conditions, and some idea of the intracellular events which follow membrane ingestion.

In this chapter, we shall review the main features of membrane uptake and intracellular transformation in the giant amoeba *Chaos chaos,* and shall then describe what is known about the system as a whole in relation to ionic and osmotic regulation during normal growth. The work on which this account is based was done in collaboration with Dr. Carl Feldherr, Dr. Vivianne Nachmias, and Dr. David Bruce, at the University of Pennsylvania. While the specific results may be relevant only to the free-living amoebae, certain concepts derived from the analysis of the system as a whole may provide a key to some of the broader problems of transport physiology.

THE CYCLE OF MEMBRANE UPTAKE AND RENEWAL

In *Chaos chaos,* both pinocytosis and phagocytosis seem to depend upon the same fundamental mechanisms, and the vesicles formed in both processes undergo apparently identical transformations within the cytoplasm. The rate at which the cycle operates is not fixed, but varies greatly according to the physiological state of the cell. In fasting cells under normal environmental conditions the turnover of surface membrane occurs only at a very low rate, as we shall see, even though the cells are actively moving and continually changing shape. A stimulus to the surface coat of the membrane by a suitable food organism, or by the application of any of a variety of cationic-inducing substances (including small cations in appropriate concentrations, and positively charged proteins, dyes, or colloidal particles) sets off the active process of membrane engulfment. Pinocytosis channels or food cups are formed, the continuity of the vesicle membrane with the surface membrane is broken, and continuity is reestablished in both by what seems to be an instantaneous reaction.

The surface membrane or plasmalemma of the amoeba is a composite structure, consisting in fixed specimens of an inner trilaminar or "unit" membrane about 100 Å thick, an intermediate amorphous coat about 300 Å thick, and an outer filamentous "fringe" which is about 1000 Å in thickness [11, 3, 2]. The coat material consists in the main of an acid mucopolysaccharide, containing about 5% of sulfate, and the sugar is a polymannose. The slime coat has been shown to contain the binding sites for the cationic inducers of pinocytosis, and to play an analogous role in phagocytosis [6]. Its cation exchange capacity has been demonstrated [9]. Unpublished work sug-

gests that it is the physical state of the slime coat which controls the osmotic permeability to water of the composite membrane.

The rate of membrane uptake and the amounts of water, food, and various solutes taken up under defined physiological conditions have been estimated for *C. chaos* on the basis of measurements on single cells and mass cultures [9]. The most useful condition to consider is the quasi-steady state of optimal growth. In 24 hours, during which time the cell doubles in mass, the average amoeba consumes about 150 paramecia. These are digested intracellularly in food vacuoles or vesicles, and the products of digestion are absorbed and utilized as the sole source of materials for synthesis and energy metabolism. Taking as a standard unit of growth the period required to double the cell mass, and considering the system to operate uniformly and continuously (an assumption which can be shown to be justified for the present purpose) we find that 70% of the cell surface membrane is taken in per hour. The corresponding fluid uptake is 40% of average cell volume per hour. The net increase in cell volume is only 3% per hour, and the bulk of the water taken up during feeding is excreted by the contractile vacuole system, which keeps pace with normal variations in fluid uptake to maintain the water content of the ground cytoplasm at a very nearly constant level.

The rate of activity of the system during rapid growth must be compared with the two extremes, the rate of uptake in the fasting or "basal" state, and the rate during brief periods of maximal stimulation, to demonstrate the range of homeostatic regulation.

In the absence of any specific stimulus to feeding or to pinocytosis, the cycle of membrane transport is shut down almost completely, even though the cells are actively moving. The amounts of fluid and of membrane taken in are so low that it is difficult to estimate them, but we believe that they come to about 1% per hour of cell volume and surface area. Cells in the basal or fasting state lose weight by about 6% per day [12], but can survive for 2 weeks or more.

The maximum rate of membrane uptake occurs under conditions which cannot be maintained for more than a few minutes, but properly prepared amoebae can feed very rapidly for about 5 minutes, and in that period will consume an area of membrane equivalent to that of their entire surface. (They must pay out new membrane rapidly in order to do this, and we shall return to that problem a little later.) During the burst of activity, the rate of membrane uptake is about 17 times greater than that during optimal growth, or 1200 times the

basal rate. After such a burst of activity, the cell requires a long period to restore the internal balance of the steady growth state.

During normal feeding and growth, the uptake of surface membrane is closely matched by the formation of new surface membrane. This process has been studied by labeling techniques and electron microscopy by Dr. Nachmias. It appears that membrane expansion occurs by the interpolation of both lipid and mucopolysaccharide materials directly from the ground cytoplasm, rather than by the splicing in of preformed cytoplasmic membranes. This is a key issue in the analysis of the system as a whole, because it follows that the ingested membrane, given a rate of uptake of 70% of the cell surface per hour, must either accumulate progressively within the cell or be broken down into a dispersed, presumably micellar, state and reutilized. Accumulation of this order clearly does not occur, nor can the membrane taken up be accounted for by the very small fraction lost in defecation balls. From such balance considerations it must be concluded that the greater part of the membrane ingested is broken down intracellularly. From other studies which we shall not attempt to describe, it has been estimated that the intracellular pool into which membrane disappears and from which new membrane forms comprises 9 to 10 times as much material as is to be found in the surface membrane itself at any one time.

The pool from which membrane forms appears to be located in the ground cytoplasm, rather than in any separate phase. Working with preparations of the ground cytoplasm isolated by direct centrifugation, we have found that membranes in the form of vesicles are readily formed *de novo* from optically clear preparations. Under slightly different conditions, similar preparations of the ground cytoplasm will also form fibrils or microtubules *in vitro*, as shown by negative staining [9]. Although the vesicles, fibrils, and microtubules which appear in such preparations are morphologically distinct, each may represent a slightly different state of the same lipoprotein material.

THE INTRACELLULAR TRANSFORMATIONS OF VESICLES FOLLOWING UPTAKE

Before experimental studies had been done on the intracellular fate of pinocytic and phagocytic vesicles, one commonly heard two views expressed concerning the physiological significance of membrane uptake, both based largely upon a priori reasoning. According to the

first, vesicles passed through the cell, their membranes ultimately re-fusing with the surface membrane. Their content was considered to be merely a part of the external environment, temporarily sequestered, and their lining membranes not to be different in structure and function from the surface membrane. Such a system, although it might be useful to transfer some materials in bulk, seemed ill-suited to perform any of the highly selective functions which interest physiologists.

The alternative view was that such vesicles represented a direct route of entry into the cytoplasm. It was postulated, for example, by Bennett [1], that the vesicle membranes broke down and the content of the vesicles was released into the cytoplasm. By this means, according to its more enthusiastic partisans, pinocytosis provided the basic mechanism of active transport.

Our experience with the amoeba has shown that neither of these views is adequate. The events which take place within the cell following membrane uptake must be described as a complex sequence of transformation in both form and function. By feeding into the system a variety of different tracer substances which can be detected by electron microscopy or by light microscopy, it has been possible to follow the marked vesicles for many hours and to deduce something of the changes which occur within the cytoplasm.

Changes in Membrane Permeability

Chapman-Andresen and Holter showed that ^{14}C-glucose is rapidly metabolized once it enters the vesicles, even though it does not pass through the external cell membrane [5]. In parallel with this change in membrane permeability, we have found that there is a great increase in the permeability of the membrane to water. To estimate the magnitude of the change, it was necessary first to determine the true permeability to water of the external membrane, i.e., the permeability of the cell in the absence of active movements of membrane. This is in fact a more difficult problem than has been supposed, since most techniques do not distinguish between the entry of water attributable to membrane permeation and that resulting from membrane activity.

In *C. chaos,* active movements of membrane are suppressed when the cell is cooled to 3°C. At that temperature the osmotic permeability coefficient is $10^{-3}\mu$/atm/min, a value 10 times less than that obtained on active cells at normal temperature (Løvtrup and Pigón, [8]). The

difference is too great to be explained entirely by the effect of cooling on diffusion, and we therefore deduce that the apparent permeability to water of the cell membrane at 25°C depends in part on active membrane movements, including the low basal level of pinocytosis already referred to.

The true permeability to water of the surface membrane in the amoeba is very low, compared to that of most cells; yet the food vacuoles or vesicles undergo a rapid reduction in volume which results in a concentration of the vesicle content by more than 10-fold. From measurements of membrane area and of the volume changes in living cells, supplemented by the results of electron microscopy, we calculate that the rate of shrinkage during the first 10 minutes implies a 100-fold increase either in permeability or in pressure. Taking into account the large initial size of the vesicles (up to 180 μ in diameter) and their irregular shape during the shrinkage process, there seems to be no mechanism capable of producing an increase in pressure of such magnitude. We therefore have concluded that the membrane becomes very much more permeable once it is taken into the cytoplasm.

During the period of rapid permeability change, the structure of the composite membrane, as seen by electron microscopy, is modified. The mucopolysaccharide coat, which initially lined the inner surface of the vesicle, breaks up or is digested, but the unit membrane part of the composite structure appears unaltered.

The Changes Associated with Digestion

Over a period of hours, digestion takes place within the food vacuoles. Changes in the structure of the individual particles of ferritin, when these are taken up in pinocytic vesicles, suggest that digestion occurs within these vesicles as well. There is no direct evidence to indicate by what route digestive enzymes enter the vesicular phase, but gold particles and ferritin, when injected directly into the ground cytoplasm, are subsequently found within digestive vesicles, even though the reverse movement does not occur. During the digestive period, many of the large vesicles divide, and there are many fusions between vesicles as well. These can be demonstrated by labeling vesicles with gold particles or ferritin. From the morphological evidence, it appears that additions to the primary vesicles occur, not only by the fusion of the separate membranes of different vesicles, but also by the engulfment or enfolding of smaller vesicles within the primary

vesicles. This process leads to the formation of complex polyvesicular bodies, but there is no difficulty in recognizing the relationships when labeling techniques are used.

The Fates of the Vesicular Membrane and of the Solutes Contained

The membrane of the primary vesicle does not break down or disappear even though its permeability to water and to some solutes is increased, and even though a large part of the membrane originally present is removed by a process we shall consider a bit later. When we trace the fate of vesicles containing ferritin or variously coated gold particles, it is invariably found that particles of macromolecular size (about 100 Å) do not escape into the ground cytoplasm, even over periods as long as 96 hours.

During that time the total surface area of the primary vesicle is greatly reduced. Reduction occurs by a remarkable process of "budding" in which small secondary vesicles form singly or in short chains at the cytoplasmic surface of the primary vesicle. Whereas the primary vesicles vary in diameter from 10 to 180 μ, approximately, the secondary vesicles are about 40 to 100 mμ in size. The process of budding appears to be quite different from that by which the primary vesicles are subdivided and fused on the larger scale. The secondary vesicles are not only much smaller, but they never contain the dense residual masses which are characteristic of the primaries, however much the latter have been subdivided or fused.

Apart from the differences in size and appearance between the primary and secondary vesicles, there are compelling reasons for considering the two as different populations with different fates. The primary vesicles evolve into dense bodies which are ultimately defecated, whereas the secondary vesicles must ultimately disappear. The fraction of the membrane originally ingested which remains with the primary vesicle is not returned to the pool, but that which is budded off to form secondary vesicles (some thousands of which are formed from each primary vesicle) does return to the cytoplasmic pool.

Solutes taken up in the original pinocytic or phagocytic vesicles are likewise partitioned between the two phases—the primary and secondary. Most tracer substances fed into the system, in the experiments we have done, were retained in the primary vesicles, and finally defecated. This included ferritin, methylated ferritin [10] and several types of gold sols the particles of which were coated or stabilized with different polyelectrolytes [7]. Such macromolecular particles did

not appear at all in the secondary vesicles even though they were randomly distributed when the primary vesicles underwent the coarser type of fusion and subdivision already described. There was, however, one interesting exception. Gold sol particles coated with polyaspartic acid behaved differently, in that they passed readily from the primary into the secondary vesicles. We have no evidence to indicate why particles coated with polyaspartic acid are handled differently from particles of similar size but different surface chemistry, but the result suggests that the content of the small secondary vesicles is determined by surface chemical forces at work during the budding process. If this is so, the system as a whole should be thought of as one which is capable of separating specifically the solutes contained in the original vesicle, of determining which shall enter the secondary vesicles and ultimately the cytoplasm, and which shall be excluded from entry, and ultimately defecated.

IONIC AND OSMOTIC REGULATION IN *CHAOS CHAOS*

From the description already given, it should be evident that any analysis of water and ion movements must take into account not only the properties of the surface membrane but also the functions of this dynamic intracellular system.

Dr. David Bruce and I have recently studied the relationships between the inside and outside levels of the principal ions of the cytoplasm, which in *C. chaos* are K, Na, and Cl. Direct analyses were done on samples of the ground cytoplasm, and the results were related to studies of electrical potential differences and of the resistance and rectifying properties of the surface membrane under different conditions [4]. For the purpose of this volume, it seems appropriate to consider only the general features of regulation suggested by the results.

In the absence of active membrane uptake, the composite surface membrane is essentially impermeable to anions but permeable to both K^+ and Na^+, which exchange passively. In the cold, when the contractile vacuole system as well as the system of membrane uptake is shut down, the cell does not discriminate between K^+ and Na^+. Under such conditions, it appears that the cytoplasmic cation level, the sum of $[K]_{in}$ plus $[Na]_{in}$, is determined by a Donnon distribution, since the ground cytoplasm contains an excess of anionic charges on the polyelectrolyte constituents, as well as Cl^- and some $PO_4^=$, and since the membrane is essentially impermeable to all anions studied

so far. Because contractile vacuole activity is suppressed in the cold, the cell swells by about 1% of cell volume per hour, as water enters osmotically through the static surface membrane.

When the cell is returned to normal temperatures of 20° to 25°C, ionic and osmotic regulation are restored, as the two systems of membrane uptake and contractile vacuole output become effective. In the basal and active states described earlier, the cell maintains the composition of the ground cytoplasm in respect to water, K^+, and Cl^- by varying the rate of contractile vacuole activity to match the varying rate of uptake. This is achieved by excreting water with Na^+ and some anion not yet determined (we suspect this may be $PO_4^=$, or some of the waste products of metabolism, or both). As a result, the level of Na^+ in the cytoplasm is normally very low (0.1 to 0.3 mM) compared to that of K^+ (30 to 23 mM).

From the electrochemical point of view, the complete system requires a minimum of two active components of "pumps." One is provided by the contractile vacuole subsystem, which maintains osmotic homeostasis and in doing so excretes Na^+. The other is required to account for Cl^- accumulation against an apparent electrochemical gradient.

We know almost nothing about the molecular mechanisms which underlie the operation of the contractile vacuole system, but from morphological evidence there is little doubt that the operation depends upon a series of intracellular transformations of vesicular elements, which appear *de novo* from the ground cytoplasm and fuse to form the contractile vacuole proper during each cycle of excretion. The over-all rate must be a function of the rate at which the smallest vesicles form from the ground cytoplasm, and this in turn must be determined by the rate at which water enters the cytoplasm, if we are to explain the range of homeostasis observed. In this instance, the "pump" is clearly not a discrete mechanism localized in the surface membrane, but a process based on the colloidal properties of the ground cytoplasm.

With regard to the second pumping mechanism required by the electrochemical evidence, we have come to a similar conclusion. The level of Cl^- in the cytoplasm (18 to 20 mM) is maintained even in the cold, and Cl^- is accumulated during cell growth, yet we find no evidence of any Cl^- pump in the surface membrane. We must ask therefore whether the entire requirement for Cl^- could be supplied by the route of pinocytic and phagocytic uptake.

Since we know the volume and ionic composition of the fluid taken in during cell growth, the number of food organisms consumed and the ion content of each, it is possible to draw up a balance sheet. Table I gives the amounts of each of the three ions, Na^+, K^+, and Cl^-, taken in by an average-size amoeba (volume $33 \times 10^6 \mu^3$) during one growth cycle, during which the cell doubles in size. The total uptake of each ion is compared with the requirement for each.

From the table it can be seen that there is a reasonable correspondence between the total Cl^- uptake and the amount required to double the cytoplasmic mass and volume. Both Na and K are also taken up in adequate amounts, but because these ions might also be supplied by passive exchange across the cell membrane, we are less concerned

TABLE I. *Ion Uptake Compared to Ion Requirements during the Growth of Chaos chaos, Feeding on Paramecium aurelia*[a]

	Na	K	Cl
Ion content of fluid ingested	3	15	30–45
Ion content of paramecia ingested	14	95	14–18
Total uptake	17	110	44–63
Requirement for growth	1–2	90–100	60–67

[a] Expressed in moles $\times 10^{11}$.

about their uptake. We conclude that the amoeba is entirely dependent on membrane uptake to supply its requirement for chloride ion. The situation with regard to $PO_4^=$ is probably similar, but this remains to be worked out.

The general concept suggested by the studies we have described is that all active transport processes in the amoeba are intracellular and depend upon dynamic transformations of membrane into cytoplasm, and of cytoplasm into membrane, rather than upon the operation of localized structures within an otherwise static "cell membrane."

There are several ways in which this concept can be further explored in free-living cells, but it would be most interesting to determine whether the transport systems operating in higher organisms—for example, in specialized epithelial tissues—are not similarly organized.

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