Protozoan Nutrition and Metabolism

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Protozoa are found in almost every type of moist habitat, a range that encompasses salt and fresh waters, soil, host organisms and environments with extremes in temperature. Their success is due in part to the ability of different protozoa to use diverse and often changing nutritional sources.

Kinds/Modes/Processes

Most protozoa are heterotrophic, requiring external organic compounds as their carbon source. They may be free-living protozoa acquiring organic molecules or organisms as food, symbiotic protists maintaining a mutually beneficial relationship with another organism, or parasites depending on a host for nutrients on a temporary or permanent basis. Some protozoa are mixotrophic organisms that use a combination of photosynthetic carbon fixation and acquisition of environmental organic material. These include flagellates with chloroplasts as an integral component of the cell structure, heterotrophic protists that temporarily retain the chloroplasts of ingested phytoflagellates, and protozoa that have endosymbiotic relationships with a cyanobacterium or green alga. In some cases, the protozoan and endosymbiont can exist independently; in others, the association is obligatory for the survival of both. A few flagellates are obligate autotrophs but have mixotrophic or heterotrophic relatives.

Nutrient assimilation

Protozoa use several routes for assimilating nutrients. Solid food is ingested by phagocytosis. Dissolved nutrients and ions may be present in fluid ingested with particles during phagocytosis, may be taken up by pinocytosis or may be transported across the cell membrane by diffusion or active transport.

During phagocytosis, solid particles, usually small organisms, are enclosed in a nascent vacuole at the cell surface. This vacuole or phagosome is separated by membrane fission and released into the cytoplasm. In protozoa delimited by a single membrane, phagosomes commonly form by invagination of the cell membrane or by the protrusion of pseudopods around the food in a manner similar to that displayed by phagocytic metazoan cells. Phagosome formation may occur over the cell surface, but more commonly the site is limited to a particular region. The naked amoeba *Acanthamoeba* collects bacteria bound to membrane receptors into a cap and subsequently transports them to the posterior end of the cell where phagocytosis takes place, while phagotrophic flagellates may use water currents produced by beating flagella to direct food particles toward the site of phagosome formation. Heliozoa capture protozoa and small metazoa by adhesion to long, thin axopods that extend from the cell surface. The axopod is retracted into the body, and the prey is enclosed by pseudopodia. In testate amoebae and foraminiferans, the site is determined by the presence of a pore in the external covering, through which ingestion takes place.

The site of phagocytosis may be dictated by the organization of the cell cortex. The plasma membrane of euglenoid flagellates is strengthened by protein strips and microtubules organized to produce alternating ridges and grooves in the surface. This pellicle is absent from the anterior pocket (= ampulla or reservoir) from which the flagella originate. In the phagotrophic members, food is ingested through the cytostome in this pocket. Armoured dinoflagellates also have pellicles or thecae in which the cell membrane is supported by cellulosic plates enclosed in membrane sacs or alveoli. Dinoflagellates have evolved different strategies for phagocytosis that include the capturing of particles by pseudopodia extended from the flagellar groove or penetrating the prey by projection of a tentacle or peduncle through which the cytoplasm is removed.

Ciliates are bounded by a pellicle formed by the cell membrane and two additional membranes, the outer and inner membranes of the alveolar sacs. This arrangement precludes the incorporation of large areas of the cell membrane into the phagosomes. Those ciliates in which phagocytosis occurs have developed oral structures to collect and direct food to the nascent vacuole. The design of the oral apparatus reflects the type of food to be

ingested. Ciliates that feed by filtering particles from the surrounding fluid have strategically placed oral ciliature to trap particles and force them toward the nascent vacuole. These ciliates are capable of rapidly moving large volumes of water through the oral region. Herbivorous and carnivorous ciliates often use the cytoskeletal components underlying the oral membrane to draw in the food. The vacuole enlarges by fusion of vesicles at the cytostome. Not all protozoa ingest their food intact. For example, suctorian ciliates rupture the membrane of their prey by discharging haptocysts from their tentacles upon contact and ingest the cytoplasm.

In some protozoa, digestion of food may begin prior to separation of the phagosome. *Tetrahymena* secretes acid hydrolases into the medium, and acid phosphatase is present in the vacuole before lysosomal fusion. In *Pseudomicrothorax dubius*, which ingests long cyanobacterial filaments at rates up to 15 μm/s (Hausmann and Peck, 1979), vesicles containing hydrolytic enzymes fuse with the nascent vacuole and the cell wall is digested, producing a flexible filament.

The details of vacuole processing have been documented in the ciliates *Paramecium* and *Tetrahymena*. The vacuole separates from the cytostome by membrane fission and moves away from the oral region (**Figure 1**). These processes are assisted by microtubules and filaments of the cytoskeleton and may be triggered by changes in the local concentration of calcium. The first step in vacuole processing is acidification by proton pumps delivered in acidosomes, which begin to accumulate around the nascent vacuole and fuse shortly after its separation. The vacuole decreases in size as vesicles are removed and recycled to the oral region. Fusion of lysosomes with the acidified vacuole introduces digestive enzymes. Late in digestion, the pH rises, and vesicles containing acid phosphatase (and presumably other hydrolytic enzymes) are removed. The final step is fusion of the spent vacuole with the cytoproct, a specialized region on the cell surface, releasing residual material into the surrounding environment. These ciliates have a mandatory processing period, ranging from about 20 min in *Paramecium* to 100 min in *Tetrahymena vorax*, before the vacuoles are capable of fusion. After defecation, the vacuole membrane is retrieved in vesicles that form at the cytoproct.

Pinocytosis (nonselective or bulk-phase endocytosis) and receptor-mediated endocytosis are pathways for the uptake of dissolved nutrients. While formation of endocytic vesicles at the cell surface has been observed in a number of protozoa, the process has been investigated in only a few. Amoebae display constitutive endocytosis representing the intake of about 1% of the cell membrane per hour in *Chaos* (Bruce and Marshall, 1965) up to a rate equivalent to turnover of 2–10 times the entire cell surface per hour in *Acanthamoeba* (Bowers and Olszewski, 1972). Induction of endocytosis by a change in pH or by the addition of chemical stimuli, notably cations and positively charged molecules, results in development of fine channels projecting from pseudopodia and phagosomes from which vesicles are released into the cell interior. Endocytosis in ciliates occurs at points in the pellicle where the outer and inner alveolar membranes join. These sites, located at the base of the cilia, have a parasomal sac where the cell membrane penetrates into the cytoplasm and terminates in a coated pit. *Paramecium multimicronucleatum* has been estimated to have about 8500 such coated pits in the cell body and buccal cavity (Allen and Fok, 2000). Coated pits also have been observed in the membrane of nascent phagosomes in *Tetrahymena* incubated with cationized ferritin (Mislan and Smith-Somerville, 1986). Vesicles formed from coated pits are processed in a manner similar to that of clathrin-coated vesicles in mammalian cells. The clathrin coat is shed soon after release, and the vesicle fuses with an early endosome, typically composed of one or more flattened cisternae with coated ends located near the basal body. Movement of carrier vesicles from early endosomes to acidosomes has been demonstrated in *Paramecium multimicronucleatum* and presumably occurs in other organisms as well.

Ions and small molecules are translocated through the cell membrane by diffusion or active transport. The specific transport pathways vary with the protozoan. Attempts to introduce labelled molecules into cells have shown that some protozoa such as *Tetrahymena* readily assimilate a large variety of molecules, metals, vitamins, sugars, amino acids, bases and nucleosides, while it may be difficult to label the desired component in other protozoa.

The degree to which each of these processes contributes to the overall nutrition varies with organism, habitat and food availability. For example, phagotrophy is important for food acquisition in *Tetrahymena*, but it is not mandatory since vacuole-less mutants grow in nutrient medium enriched with trace metals and vitamins. Endocytosis and membrane transport of dissolved solutes probably account for much of the nutrient assimilation in symbiotic protozoa, although some do carry out phagocytosis as well.

Energy production

With the exception of a few parasites, protozoa maintain internal stores of polysaccharides. The most common storage form is glycogen, but exceptions include paramylon in euglenoid flagellates and amylopectin in rumen ciliates, gregarines and *Eimeria*. In contrast, *Plasmodium* and trypanosomatid flagellates, which have ready access to glucose from the host, do not store polysaccharide.

Glucose, assimilated or derived from stored reserves, is the major source of energy for protozoa. Most protozoa are capable of catabolizing glucose to pyruvate by glycolysis. In oxygen-poor environments, pyruvate can be degraded to one of several end products by fermenta-

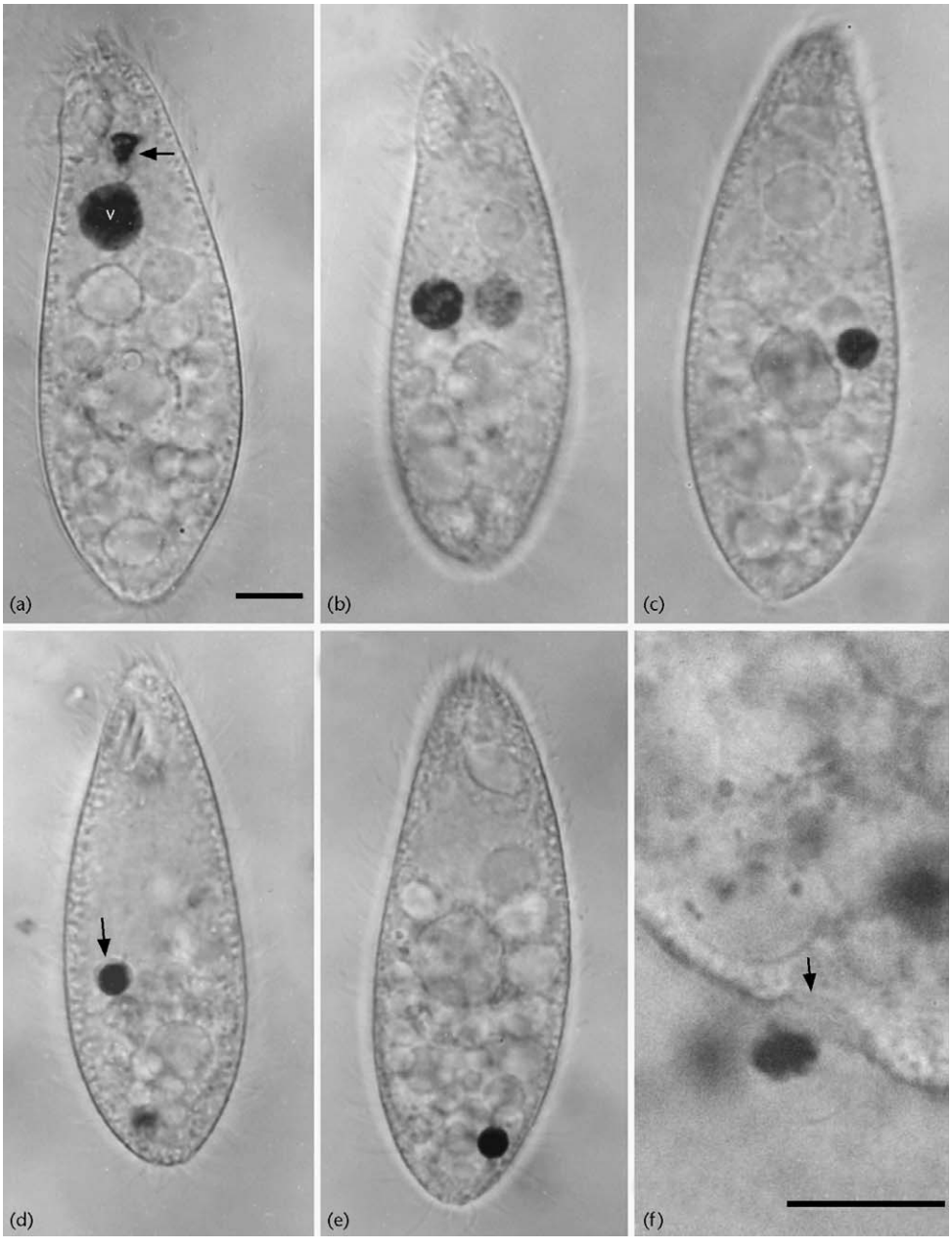

Figure 1 Series of light micrographs showing progress of vacuoles through the digestive cycle in the ciliate *Tetrahymena vorax*. The vacuoles contain particles of India ink, which are not degraded by digestive enzymes. (a) Nascent vacuole attached to cytostome (arrow) and newly formed vacuole (V) near oral region. (b) Two vacuoles undergoing acidification. The decrease in the diameter of the vacuoles condenses the contents into a compact mass. (c) A vacuole at a time when the pH of the vacuole is the most acidic. (d) A vacuole after fusion with lysosomes. The addition of the lysosomal membrane results in a clear region between the compact India ink mass and the vacuole membrane (arrow). (e) A vacuole at the posterior end of the cell late in the digestive cycle. (f) A mass of India ink particles recently released from the cytoproct (arrow) at the posterior end of the cell. Bars = 10 μm. Reproduced with permission from Mislan and Smith-Somerville (1986).

tion. When oxygen is present, most aerobic protozoa convert pyruvate to acetyl-CoA used in the tricarboxylic acid (TCA) cycle for generation of adenosine triphosphate (ATP) by oxidative phosphorylation in the mitochondria. *Trypanosoma brucei* uses mitochondrial respiration when in the tsetse fly, the vector for this parasite; however, the trypomastigote in the bloodstream of the vertebrate host oxidizes glucose only to pyruvate, which is excreted as the end product. Enzymes of the glycolytic pathway are compartmentalized in the glycosome, an organelle com-

mon to kinetoplastid flagellates. Mitochondria in the bloodstream trypomastigote lack cristae, but these appear during transition to the procyclic trypomastigote in the intestine of the tsetse fly. Trichomonad flagellates, rumen ciliates and certain free-living ciliates that inhabit oxygen-poor environments have hydrogenosomes. These organelles use pyruvate imported from the cytoplasm to produce ATP with the generation of molecular hydrogen. Hydrogenosomes are distinct from mitochondria, but the two organelles are considered to have arisen from a common ancestor.

Organic acids such as acetate, short chain fatty acids, amino acids or alcohols are acceptable carbon sources for acetate flagellates, a group that includes kinetoplastid flagellates and the closely related euglenoid flagellates. In some cases, these substrates represent alternative carbon sources to glucose. Others are unable to process glucose and utilize one or more of these substrates instead.

Peroxisomes are common in protozoa, and in some organisms including *Tetrahymena*, these organelles contain enzymes of the glyoxylate pathway for conversion of fatty acids to carbohydrate. Protozoa in the phylum Apicomplexa contain an apicoplast, a remnant of an endosymbiont chloroplast, with enzymes for the biosynthesis of fatty acids, isoprenoids and haem.

Food/Energy Sources

Protozoa may be generalists or specialists with regard to their natural diet. Bacteria constitute the main food for many phagotrophic protozoa including flagellates, ciliates and small amoebae (**Figure 2**). *Stentor* and related ciliates are omnivores, ingesting not only bacteria but algae, ciliates, flagellates and microscopic animals such as rotifers as well. Other protozoa are more selective. Herbivorous organisms include the ciliate *Nassula* and the large amoeba *Pelomyxa* which feed on diatoms and algae. Certain ciliates such as *Grossglockneria* feed solely on fungi. Carnivorous protozoa range from those that feed on a variety of prey such as ciliates, flagellates and rotifers to those with a preference for a particular organism. The latter case is illustrated by *Didinium nasutum*, which prefers *Paramecium*. Some protozoa have adopted a scavenger lifestyle, feeding on gut contents or exudates of animal hosts. Intestinal flagellates of termites and woodroaches provide particularly intriguing examples of protozoa that ingest gut contents (**Figure 2**). The host is dependent on these symbiotic protozoa for digestion of wood and cannot survive on a diet of cellulose without them. Decomposing organic matter provides a rich source of dissolved nutrients, particularly for those free-living protozoa that rely on uptake of fluid and molecular transport for nutrient assimilation. The protozoa with the highest degree of dietary specificity are parasitic forms that use particular

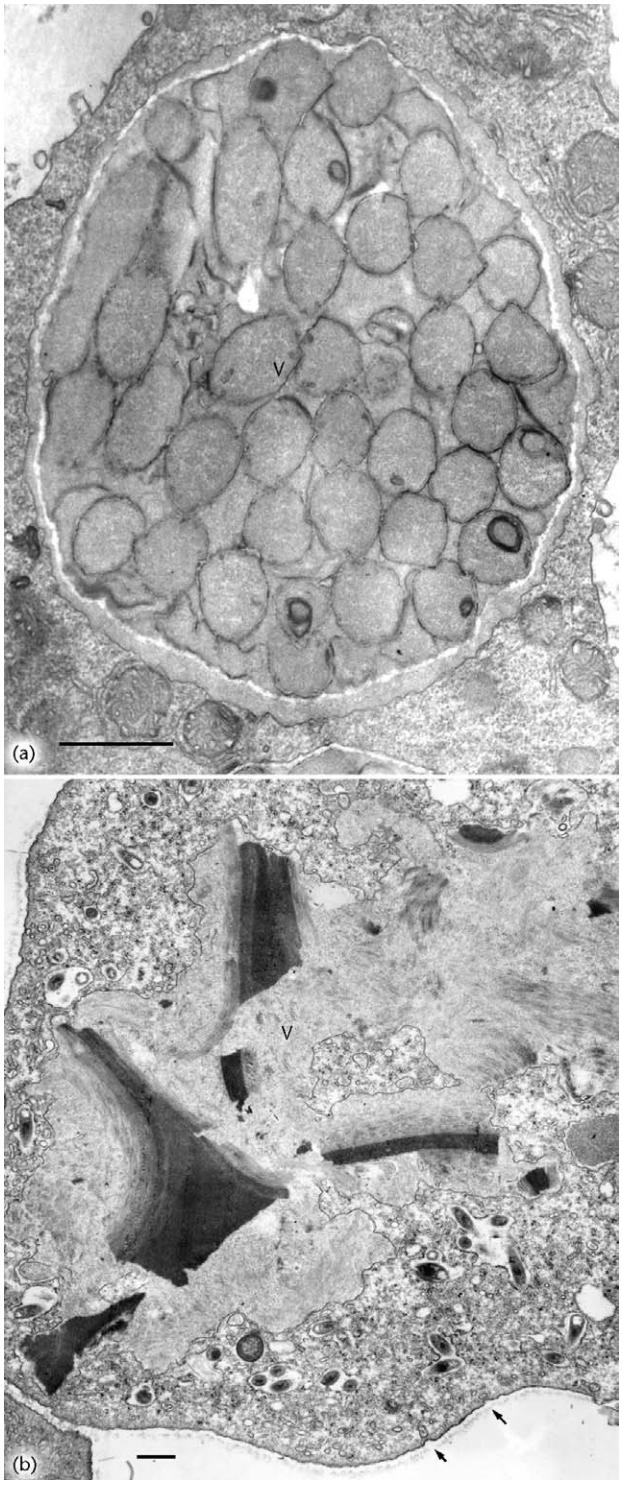

Figure 2 Electron micrographs of vacuoles with ingested food. (a) Partially digested bacteria in vacuole (V) in the ciliate *Tetrahymena vorax* after fusion of lysosomes with the phagosome. (b) Wood in vacuole in *Pyrsonympha vertens,* an intestinal flagellate of the termite *Reticulitermes flavipes*. Arrows indicate sites of endocytosis at the cell surface. Bars = 1 μm.

body fluids or live inside specific cells. These protozoa are often difficult to maintain in culture without the host.

The nutritional needs have been defined for protozoa that can be grown in axenic culture. A small number of protozoa have only a few requirements; others must be supplied with various ions and organic molecules. The precise composition often differs even among species of the same genus. Those with chloroplasts or photosynthetic endosymbionts usually require few or no organic supplements when light is adequate. Mixotrophic species with chloroplasts, such as many euglenoid flagellates, may require only salts and trace metals in the presence of light, but can grow without light if acetate is added as a carbon source. Most protozoa have more extensive requirements. In addition to glucose or an acceptable substitute such as other saccharides or acetate, the list of components may include amino acids, nitrogenous bases, lipids, vitamins and essential elements supplied as inorganic salts. One amino acid to over half of the 20 amino acids may be essential for growth in various species; the remaining amino acids can be synthesized from precursors by the protozoan. In addition to serving as monomers for protein synthesis, amino acids can contribute to energy production as precursors to components of the TCA cycle, and in some cases may be used instead of glucose or other saccharides. One or more bases are mandatory requirements for those protozoa that lack the enzymes for *de novo* synthesis of purines and/or pyrimidines. *Plasmodium falciparum* cannot synthesize purines, while *Tetrahymena* requires inclusion of both a purine and a pyrimidine in chemically defined culture medium. Addition of lipids, fatty acids or sterols is an absolute requirement in only a few cases, but may enhance growth if included. β-Oxidation of lipids can provide a supplemental source of energy.

Physiochemical Factors

Food selection

For many protozoa, food selection is based on abundance and ease of capture, and feeding rates will change with food concentration. This is most evident in ciliates and flagellates that filter the food from water currents generated by their cilia or flagella. Although bacteria constitute the primary food of these cells, the major factor for uptake is often particle size and not nutritive value. *Paramecium* and *Tetrahymena* ingest particles of India ink, carmine, latex spheres and ferric oxide as readily as bacteria, a property that has been exploited to investigate phagosome processing (see **Figure 1**).

Many protozoa use physical stimulation and/or chemoreception to locate food. Prey mobility may result in initial attraction by producing currents sensed by the predator at a distance, but chemosensory signals provide the primary stimulus for the actual phagocytic response. When offered a choice, the large carnivorous amoeba *Chaos carolinense* will select *Paramecium* for ingestion over *Tetrahymena* and *Euglena*, while *Amoeba proteus* prefers *Tetrahymena*. The stimulus for this selection is the interaction of a labile substance with receptors on the surface of the amoeba. Pseudopod formation can be induced by living or killed prey as well as a variety of dissolved factors ranging from cytochrome *c* to trypsin and lecithin. The ability of phospholipids and several other substances to initiate phagocytosis has been demonstrated for certain carnivorous ciliates and flagellates. A broader range of exogenous factors stimulate pinocytosis, particularly positively charged solutes and ions.

Certain protozoa appear to depend on chance interaction for feeding. Phagotrophic sessile organisms that lack the ability to move toward the food often depend on random contact, but feeding may still be selective, with only certain organisms invoking the phagotrophic response.

Certain protozoa can respond to food availability by altering their morphology, making it possible to ingest alternative types of food. *Blepharisma* and polymorphic species of *Tetrahymena* have small oral structures when bacteria are plentiful, but replace this oral apparatus with one of larger dimensions when other ciliates represent the only abundant prey (**Figure 3**). In *Tetrahymena vorax*, this response is induced in the presence of stomatin, the water-soluble exudate of prey ciliates. The active signal in stomatin is a chelate of ferrous iron, hypoxanthine and uracil (Smith-Somerville *et al.*, 2000). Large quantities of these bases are released into the medium as the end products of purine and pyrimidine degradation, but in their free molecular forms they do not appreciably stimulate the morphological change.

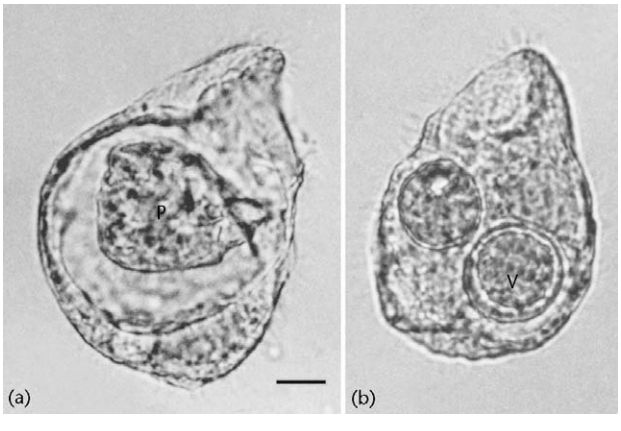

Figure 3 Macrostomal cells of the polymorphic ciliate *Tetrahymena vorax*. The microstomal form (shown in **Figure 1**) differentiates into this cell type in response to a signal released by potential prey. (a) A prey protozoan (P) in a vacuole before separation from the cytostome. (b) Two vacuoles (V) with protozoa in different stages of digestion. Bar = 10 μm.

Chemical signalling

Chemical sensing in protozoa extends beyond detection of food. It is often desirable to generate uniform cultures by cloning single protozoa. In some cases, production of cultures from individual cells is efficient only when they are placed in conditioned medium – culture medium from which cells have been removed. Studies on survival of *Tetrahymena* in chemically defined medium support the hypothesis for a threshold level of a signal or signals required for cell survival. Certain molecules, including sterols, dipalmitoyl phosphatidylcholine, porphyrin, haemin, diacylglycerol, croton oil, certain proteins such as insulin, and small peptides, can obviate the need for signals released by the ciliate. The first identified growth factors are a 17 kDa protein secreted by the ciliate *Paramecium tetraurelia* (Tanabe *et al*., 1990) and a 4–6 kDa factor from *Tetrahymena thermophila* (Schousboe *et al*., 1998).

Hydrogen ion concentration

pH affects the solubility and ionization state of nutrients and influences nutrient assimilation. Optimum extracellular pH for most protozoa is around neutral, but many will tolerate a pH range of 5–8, which may be encountered in environments with diurnal and seasonal fluctuation. Acetate flagellates are tolerant of environments with greater acidity resulting from high concentrations of organic acids. Parasitic organisms may experience changes in surrounding pH depending on their location in the host. Many protozoa will encyst when subjected to adverse conditions, including pH shock, as a survival mechanism.

Oxygen and redox potential

The redox potential of the environment is determined by the concentration of reducing and/or oxidizing components, including oxygen. This factor is influenced by pH and affects not only energy production but also the availability of trace metals such as iron. Protozoan species vary in their ability to inhabit environments with different redox potentials and oxygen levels. Some protozoa are obligate anaerobes that occupy anoxic environments or habitats with very low oxygen concentrations and high levels of reducing agents. Many are either aerobic or facultative anaerobic organisms. They may have adaptations to different oxygen levels that vary with life cycle stage or may move to accommodate fluctuating oxygen levels. *Loxodes*, for example, migrates with the changing anoxic–oxic zone in lakes.

Temperature

Species of protozoa inhabit environments ranging in temperature from subzero at high salinity to 45°C or above in thermal springs. Thermophilic species have developed heat-stable proteins and enzymes with high temperature optima. Free-living protozoa that inhabit shallow waters are able to withstand daily fluctuations in temperature due to solar warming. Encystment provides a protective mechanism for many protozoa when they encounter temperatures outside the limits of tolerance.

Culture Techniques

Many protozoa have been cultured successfully in the laboratory. Free-living species are easier to grow, but the list now includes a number of parasitic species for which at least one stage in the life cycle may be maintained outside the host. *Protocols in Protozoology* (Lee and Soldo, 1992) is an excellent source of information on the cultivation of over 50 different protists. A database of media formulations searchable by particular protozoan is maintained by the American Type Culture Collection, and formulations for strains in the Culture Collection of Algae and Protozoa, the national service collection in the United Kingdom, are available on its website.

The culture medium must supply all essential nutrients. If more than one formulation can be used to culture an individual type of protozoan, the choice of medium is largely dependent on the intended experimental use for the organisms. Directions for preparing chemically defined medium with precise amounts of organic molecules and salts are available for a variety of organisms, but are often difficult and expensive to make. For general maintenance and many experimental procedures, protozoa are usually grown in a medium containing complex, often incompletely defined supplements. Commercial products for microbiological media are common nutritional additives. The medium may be supplemented with certain nonessential components to stimulate growth. It may be necessary to include live food sources such as bacteria or small eukaryotic organisms. These cultures may contain material such as rice grains to promote bacterial growth. Many parasitic protozoa are maintained in animal cell cultures or in the native host.

Culture conditions must be optimum to obtain maximum growth. These factors include temperature, pH, osmolarity, appropriate atmospheric gases, proper gas exchange, and light for protozoa that photosynthesize. Optimum temperature and pH are usually close to those found in the natural habitat for the species. Aerobic protozoa often require large ratios of surface area to volume for gas exchange. Shaking the culture may decrease the ratio, although factors such as the type of shaking and cell density influence the result. Certain ciliates become senescent with time and require sexual reproduction to maintain vigor.

In vitro cultures may be grown in a variety of vessels depending on the number of cells desired. For routine maintenance, protozoa are usually grown in culture tubes or small flasks, with larger flasks used to generate greater numbers of cells when necessary for particular studies. Cultures are started using an inoculum containing one organism or more than one. The frequency of passage depends on the inoculum, the rate of growth and the accumulation of waste products. Continuous culture apparatus that automatically replenishes nutrients has been devised for production of mass quantities of protozoa and for particular experiments.

Efficient long-term preservation methods for cell lines are as important as successful culture methods. Long-term backstocks preserve well-characterized cells and other cells of interest for future studies, and help suppress molecular changes (mutations) that accumulate over time in continuous culture. Techniques applicable to certain protozoa fail with others but, when they are successful, cells can remain viable for periods of several years. A popular method is the cryopreservation of cells at liquid nitrogen temperature. Cultures are incubated in nonnutrient buffer, infused with a cryoprotective agent (often dimethyl sulfoxide), subjected to rate-controlled cooling, and finally stored in liquid nitrogen. Freeze drying is another preservation method often applied to certain protozoa.

Chemotherapeutic and Medical Applications of Such Studies

Over 50 000 described species are classified as protozoa. Most are harmless and may play beneficial roles in the ecosystem. About 20% are parasites of humans and/or other vertebrates, invertebrates, plants or other protists. Those that infect humans and commercially important organisms can cause significant economic losses and medical problems that may result in human mortality. Some species of common free-living protozoa may be opportunistic parasites. *Naegleria fowleri*, an amoeboflagellate sometimes found in the water of ponds and swimming pools, can penetrate the nasal mucosa and infect the brain, resulting in meningoencephalitis. Several species of *Acanthamoeba* can affect humans. Although these amoebae may invade the central nervous system, the more common problem is *Acanthamoeba* keratitis often associated with use of contact lenses. Protease secretion and phagocytosis are significant factors in this pathogenesis (Khan, 2001). While not parasitic, other protozoa produce toxins that result in major environmental damage and human suffering. Although treatments for many of the diseases are available, the combination of classical morphological and biochemical approaches to understanding nutrient assimilation and metabolic processes in both pathogenic and model free-living organisms, coupled to results from genome sequencing initiatives and proteomic analyses, promises to provide potent new therapies for control.

The medical applications of studies on protozoan digestion may be indirect. The bacterium *Legionella pneumophila* enters one of several species of protozoa by phagocytosis and alters the normal digestive pathway, allowing it to survive and multiply inside the cell. The protozoan provides protection against biocides placed in air-conditioning cooling towers and plumbing systems, water systems that have been linked to outbreaks of Legionnaires' disease. Both normal phagosome processing and the survival mechanism used by *Legionella pneumophila* are similar in protozoa and mammalian phagocytic cells. Thus, the information gained from studies of phagocytosis should further our understanding of the process not only in these protozoa but in mammalian cells as well.

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