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Differentiation of pathogenic amoebae: encystation and excystation of *Acanthamoeba culbertsoni* —A model

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Abstract. Differentiation into dormant cysts and vegetative trophozoites is an inherent character intimately associated with the life cycle and infectivity of pathogenic amoebae. In the case of human intestinal amoebiasis encystation and excystation are of immediate relevance to the process of transmission of the disease from healthy carriers to susceptible individuals. Using a pathogenic free living amoeba *Acanthamoeba culbertsoni* as a model, considerable progress has been achieved in understanding the mechanism and control of the process of differentiation. The turnover of the regulatory molecule cyclic 3: '5' adenosine monophosphate is responsible for triggering the process of encystation. Amoebae bind effector molecules such as biogenic amines to a membrane localized receptor which itself resembles the β-adrenergic receptor of mammalian organisms. The activation of adenylate cyclase or inhibition of cyclic AMP phosphodiesterase maintain the dynamic intracellular cyclic AMP. The cytosol fraction of amoebae has a cyclic AMP binding protein. During encystation, enzymes needed for synthesis of cellulose and glycoproteins are induced. Control is exercised at transcriptional level and the process is subject to catabolic repression.

Excystation of mature amoebic cysts is mediated by glutamic acid and certain other amino acids by an as yet unelucidated mechanism. During excystation there is dormancy break, induction of deploymerizing enzymes *viz.* two proteases, a cellulase and a chitinase. The empty cysts or cyst walls are digested by these enzymes and their break down products are used for cellular growth. By invoking a flip-flop mechanism of repression and derepression some plausible explanation can be offered for the cascade of biochemical events that sets in when amoeba is 'turned on' to encystation or excystation.

Keywords. Amoeba; encystation; excystation; cyclic AMP.

Introduction

The phylum protozoa includes many microorganisms of relevance to human health and the environment (table 1). Thus malaria, sleeping sickness, amoebiasis, giardiasis, leishmaniasis are all human afflictions caused by protozoa and represent the phenomenon of parasitism. On the other side we have in protozoons many examples of symbiosis or biological interdependence such as in the *Mixotricha paradoxa* a protozoon which lives in the gut of the Australian termite. *M. paradoxa* is covered by thousands of spirochetes. When the latter move, *M. paradoxa* is propelled along smoothly, its own flagella being used only for steering. Another classical example of symbiosis is the protozoon *Paramoecia bursaria* which derives its colouration from the microscopic algae of the *Chlorella* genus which it harbours. The malarial parasite *Plasmodium falciparum* uses an arthropod vector, the mosquito, and the human host to

Table 1. Protozoan diseases of man.

Amoebiasis	Intestinal, Hepatic, brain	*Entamoeba histolytica*	infective cyst in faeces, invasive trophozoite in intestinal and other tissues
Primary Amoebic Meningo-encephalitis	Central nervous system	*Acanthamoeba castellanii* *Noegleria fowleri* *Hartmannella* sp.	Free-living amoebae in water, soil and decaying vegetation, as cysts; trophozoites in tissues
Ciardiasis	Intestinal	*Giardia lamblia*	Cysts or trophozoites in faeces; trophozoites in deodenal drainage
Trichomoniasis	Caecum/colon	*Trichomonas hominis*	non-pathogenic sp.—only trophozoite known (no cystic stage) in diarrhoca
	Genito-urinary tract of women urethra of men	*Trichomonas vaginalis*	motile flagellate in the discharge (trophozoite)
Trypanosomiasis African (Sleeping sickness)	Blood, lymph nodes, cerebrospinal fluid	*Trypanosoma gambiense*	infective mastigote stage in the salivary glands and mouth.
		Trypanosoma rhodesiense	*Glossina* sp. (tse-tse fly) inoculated into blood of man
American (Chagas' Disease)		*Trypanosoma cruzi*	infective metacyclic stage in the faecal droplet of the bug Triatoma contaminating its bite on conjunction mucous membranes. esp. of children.
Leishmaniasis	Visceral (Kala-azar) spleen, liver, lymph node or blood	*Leishmania donovani*	flagellate infective stage in the sand-fly, *Phlebotomus argentipes*, inoculated into blood by its bite, amostigote stage in spleen.
	Custaneous (Oriental Sore) skin and mucous membrane of nose	*Leishmania tropica L. brasiliensis*	as above, in *P. sergenti* in India, its bite (*L. brasiliensis*) in South America.
Malaria	Blood and tissues (liver, spleen, etc.)	*Plasmodium vivax* (Benigtertian) *P. malariae* (*Quartan*) *P. falciparum* (Malignant tertian) *P. ovale* (*ovale malaria*)	Transmitted by bite of various species of mosquitoes (Anopheles) sporozoites in salivary glands (infective) and trophozoite, schizont and gametocyte in man
Balantidiasis	intestinal—causing dysentery	*Balantidium coli*	cysts of the ciliate in faecas of man, contaminating soil, water, vegetables hands etc. and ingasted.
Toxoplasmosis	body tissues (esp of *Toxoplasma gonidia* the eye) intra-cellular chorio-retinitis, iritis, etc.	*Toxoplasma gonidia*	cystic stages of the coccidian in faeces of cats, trophozoite (invasive stage) in tissues of man.

complete its differentiation process the crucial steps of which have defied so far all research endeavours.

Ciliated protozoons, parasitic and free living amoebae are among the unicellular organisms other than bacteria which biochemists have used extensively for elucidating the mechanism of the chemical reactions and control processes operating behind the scene in cellular differentiation. *Tetrahymena pyriformis* has been a good model for investigations into the molecular basis of ciliary movement, adaption to unfriendly environments and the phenomenon of rapid regeneration of tissues. The free living amoebae *Acanthamoeba* have been very useful in gaining understanding of the chemical processes of encystation and excystation. An account of recent work on amoebic differentiation is presented in this review.

Definitions

The process by which amoebae lose their characteristic pseudopoidal movement and get endowed with a protective and impenetrable wall is called encystation. The resulting round bodies are called cysts. Cysts are immotile and remain metabolically inert as long as conditions are unfavourable for hatching. Once the appropriate environment for dormancy break is established, the cysts shed their coat and resume trophic or vegetative life by a process called excystation. Encystation could also be considered as a defensive posture adopted by the amoeba to overcome stresses imposed on it by a hostile environment. By analogy, excystation would constitute the emergence of the amoeba from a dormant to an active metabolic status. Transformation of trophic amoebae to dormant cysts and the reincarnation of the cysts as trophozoites (figure 1)

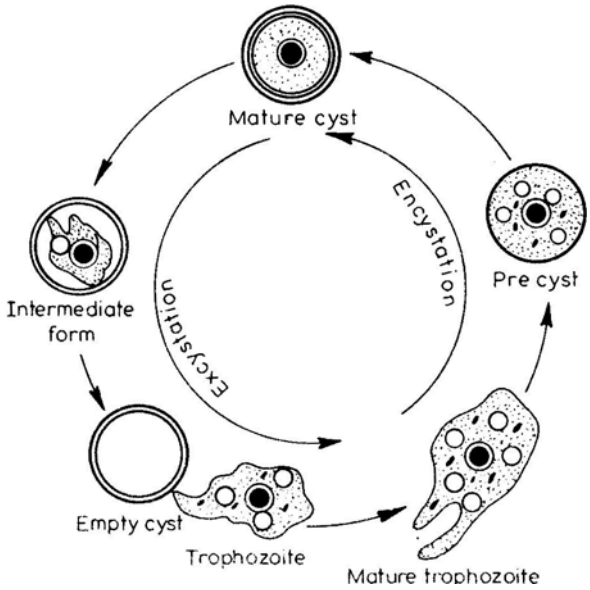

Figure 1. Schematic representation of differentiation in *A. culbertsoni.*

envisage cellular differentiation subject to many attendant control mechanisms (Trager, 1963; Willmer, 1963; Sussman, 1965; Wright, 1966; Krishna Murti, 1971,1975).

Differentiation and pathogenesis

Biochemical reactions mediated during differentiation are germane to the pathology of endemic amoebiasis caused by the human intestinal parasite *Entamoeba histolytica* and the pathology of amoebic encephalitis caused by a few strains of free living *Acanthamoeba.* With a high degree of endemicity in Egypt (85%), Equador (56%), Liberia (52 %) and India (35 %) and a global prevalence rate of 10 % amoebiasis in its intestinal and hepatic manifestations is as yet an unsolved public health problem in the Third World (Freedman, 1958; WHO, 1969). Attempts to demonstrate a toxin-linked pathogenic mechanism have been unsuccessful. At present there are no immunological guide posts for devising preventive or prophylactic strategies excepting the environmental control of the source of infection. Acute attacks of amoebic dysentery are usually amenable to management by amoebicidal drugs, such as halogenated hydroxy quinolines, metranidazole, chloroquine or some antibiotics. In contrast, chronic amoebiasis associated with the harbouring of recalcitrant cysts continues to be a clinical problem (WHO, 1969). The management of infestinal amoebiasis is complicated by the fact that many healthy persons who have never suffered from amoebiasis are carriers of infective cysts.

Amoebic meningoencephalitis due to free living amoebae has been recognised as a distinct clinico-pathological entity (Fowler and Carter, 1965; Batt, 1966; Culbertson *et al.,* 1966). Pathogenic strains of *Naegleria* have been isolated from human cerebrospinal fluid (Carva and Novak, 1968) and implicated in fatal meningoencephalitis in man (Carter, 1968; Duma, 1972, 1980). The pathogenic potential of *Hartmanella culbertsoni* now christened as *Acanthamoeba culbertsoni* is manifest as cerebral (Kenny, 1971; Jager and Stamm, 1972; Robert and Rorke, 1973; Bhagwandeen *et al.,* 1975), respiratory (Eldridge, 1967) and eye infections (Naginton *et al.,* 1974; Visveswara *et al.,* 1975). Fatal meningoencephalitis has also been reported with *Acanthamoeba* (Carter *et al.,* 1981; Grunnet *et al.,* 1981). The ubiquitous distribution of free living amoebae in fresh water, sea water, the melt water of Antartica, the latex of *Euphorbia,* soil, cultures of animal cell lines maintained in laboratories and even in samples of bottled mineral water in some regions adds yet another dimension *viz.,* environmental contamination, to the pathology associated with these organisms (Jahnes *et al.,* 1957; Culbertson, 1961; Chang *et al.,* 1962; Moore and Hlinka, 1968; Casemere, 1969; Peloux *et al.,* 1974; Singh, 1965; Visvesvara and Balamuth, 1975; Griffin, 1978; Willaert *et al.,* 1978; Jone Kheere, 1981; Rivera *et al.,* 1981).

Differentiation studies on free living amoebae

Band (1963) and Griffiths and Hughes (1968) developed replacement media to study the influence of diverse factors on differentiation. With this as the starting point and using *Acanthamoeba culbertsoni* (Singh and Das, 1970) as the test organism, extensive studies have been conducted in the Division of Biochemistry, Central Drug Research Institute,

Lucknow in the period 1969-1983 leading to the doctoral dissertations of Raizada (1972); Verma (1975), Kaushal (1976) and Srivastava (1982). Extension of the studies based on free living amoeba to axenically grown *Entamoeba histolytica* was the subject matter of the doctoral dissertation of Mitra (1975). The highlights of these investigations are summarised in the following sections.

Encystation in non-nutrient medium

More than 80 % of trophozoites of *Acanthamoeba culbertsoni* were transformed into cysts when they were placed in non-nutrient agar containing 80 mM sodium chloride, 15 mM magnesium chloride and 20 mM taurine. Encystation was inhibited by actinomycin D and cycloheximide. The following morphological changes are invariably noticed when trophozoites undergo encystation under the above conditions: reduced motility, gradual withdrawal of pseudopodia, rounding up, shrinkage in cell size and the final formation of an immotile double walled cyst (Raizada and Krishna Murti, 1971 a,b; Raizada, 1972). When cysts formed in the minimal medium are transferred to a growth medium, the cysts undergo dormancy break and release motile and vegetative trophozoites.

Encystation of *A. culbertsoni* can also be induced by incubating trophozoites in the minimal medium incorporating epinephrine, norepinephrine, tyramine or 5 hydroxy tryptamine (Verma, 1975; Verma *et al.,* 1974a; Verma and Krishna Murti, 1976a). It is not necessary that trophozoites have to be continuously in contact with taurine or biogenic amines to be 'turned on' for differentiation. Exposure to these agents for 6 h or less is all that is required for the trophozoites to become committed to differentiation.

Role of cyclic AMP in encystation

The triggering action of biogenic amines or taurine is mimicked by dibutyryl cyclic 3:5 adenosine monophosphate (Raizada and Krishna Murti, 1972a). During the transformation of trophozoites into viable cysts there is evidence for the accumulation of cyclic AMP as shown in figure 2. Taurine or biogenic amines get bound to specific receptors on the membrane of *A. culbertsoni* and activate a membrane bound adenylate cyclase (Raizada and Krishna Murti 1972a, 1973). Achar and Weissman (1980) have confirmed that increased intracellular levels of cyclic AMP are attained when *Acanthamoeba* cells in late log phase are transferred to an encystation medium. Encystation induced by taurine or epinephrine as well as the activation of adenylate cyclase are inhibited by actinomycin D or cycloheximide suggesting that regulatory

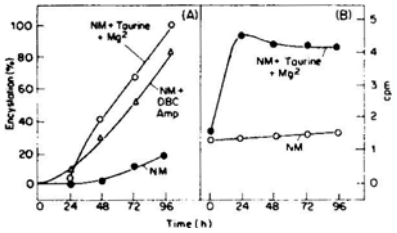

Figure 2. Differentiation of *A. culbertsoni* triggered by cAMP.

control of differentiation is exerted at the transcriptional and translational levels (Krishna Murti, 1975).

Changes in chemical composition and biosynthesis

Morphological changes observed during encystation of *A. culbertsoni* are accompanied by changes in the chemical composition and enzyme profile. The most dramatic change is the switching over of metabolism from aerobic to an anaerobic phase ending up eventually in total dormancy. Residual food materials such as glycogen and lipids are metabolized accompanied by a rapid turnover of phosphorylated compounds. During encystation there is a net loss of cellular DNA, RNA and protein but a concurrent gain in mucopolysaccharides and cellulose. However, incorporation studies with uracil-2-[^{14}C]- and valine-1-[^{14}C]- or leucine-1-[^{14}C]- indicate synthesis of new species of RNA and protein. The most interesting change noticed is the high rate of incorporation of glucose-U-[^{14}C]- into a polysaccharide which has been identified as cellulose by hydrolysis with fungal cellulase and recovery of labelled glucose from the hydrolysates. This is the first such report of the ability of a protozoon organism to synthesize cellulose which has been considered hitherto exclusive to the structure of plant cells. There was also rapid incorporation of glucosamine-1-[^{14}C]- into mucopolysaccharides (Raizada and Krishna Murti, 1972b). Preliminary evidence for the acquisition of new serological characters has also been adduced in cells undergoing differentiation into cysts (Raizada *et al.,* 1972). Substantial decreases in the contents of RNA, triacyl-glycerides, glycogen and protein have also been recorded in *Acanthamoeba* during encystation (Bowers and Korn, 1969; Neff and Neff, 1969; Rudick and Weisman, 1973; Stevens and Pacher, 1973). Solube proteins, amino acids, nucleotide derivatives and carbohydrates appear in the medium shortly after the onset of encystation (Neff *et al.,* 1964; Neff and Neff, 1969).

Cyclic AMP phosphodiesterase

Trophozoites of *A. culbertsoni* possess cyclic AMP phosphodiesterase activity (Raizada and Krishna Murti 1973). Imidazole, a known activator of cyclic AMP phosphodiesterase, did not inhibit the multiplication of *A. culbertsoni* in the axenic medium but prevented the epinephrine or taurine induced encystation of the trophozoites in the non-nutrient medium (Verma and Krishna Murti, 1975). Theophylline, a known inhibitor of cyclic AMP phosphodiesterase, could on the other hand induce encystation presumably by inhibiting the degradation of cyclic AMP. If one assumes that accumulation of cyclic AMP to an optimum 'critical' level is necessary for triggering the differentiation process, imidazole can be said to inhibit encystation by interfering with the accumulation of cyclic AMP. In contrast theophyline activates encystation by facilitating the accumulation of cyclic AMP.

Turnover of cyclic AMP

The intracellular concentration of cyclic AMP at any phase of cellular life reflects the turnover of this regulatory molecule involving the balance of synthesis by adenylate cyclase and degradation by cyclic AMP phosphodiesterase (Robison *et al.,* 1971). The activity of cyclic AMP phosphodiesterase of *A. culbertsoni* exposed to taurine or

epinephrine during starvation was significantly less than that of control cells incubating in the non nutrient medium. Some enzymic activity could be detected in the non nutrient medium whether the cells were exposed or not to taurine or epinephrine. The decay of phosphodiesterase is faster when cells are committed to differentiation triggered by epinephrine or taurine (Kaushal *et al.,* 1976). In contrast the adenylate cyclase activity is stimulated three fold under similar conditions (Raizada and Krishna Murti, 1972). Thus the stimulated adenylate cyclase and the fast decaying cyclic AMP phosphodiesterase together contribute to the build-up of relatively high concentrations of cyclic AMP required for controlling the biochemical events mediating the formation of macromolecules needed for structuring the cyst wall (Krishna Murti, 1973).

The nature of interaction between taurine or epinephrine and particulate subcellular fractions prepared from *A. culbertsoni* has been elucidated (Raizada and Krishna Murti, 1971, 1973; Verma and Krishna Murti, 1976a,b). Receptors for the binding of taurine-[^{35}S]- and epinephrine- [^{14}C]- were found to be located on the lipoprotein membranes. Subcellular particulate fractions prepared from trophozoites exposed to epinephrine prior to cell rupture exhibited a significantly higher adenylate cyclase activity than similar preparations made from cells unexposed to epinephrine. Binding of epinephrine to membraneous fractions of *A. culbertsoni* was inhibited by the drug propranalol, a well known *β*-adrenergic blocker. Interestingly enough, the same drug interfered with the induction of encystation of trophozoites by epinephrine. It may be noted that this is the first reported presence of *β*-receptors for catecholamines in amoebae.

Catabolite repression

Glucose inhibits encystation induced by taurine or epinephrine (Verma and Raizada, 1975). The glucose arising out of glycogenosis during differentiation is channeled towards the synthesis of cellulose and glycoproteins. It is likely that the glucose added from outside is exerting a catabolic repression of the induction of enzymes needed for the biosynthesis of cellulose and glycoproteins. Catabolic repression is also exerted by acetic and citric acid. Catabolic repression induced by different nitrogenous compounds indicate that both nitrogen and carbon catabolite repression is involved in encystation of *A. culbertsoni* (Srivastava and Shukla, 1983).

Polyamines and chemistry of effectors

The demonstration of a protein which binds cyclic AMP in the cytosol fraction of *A. culbertsoni* is the final event in the story of encystation (Verma and Krishna Murti, 1976b). The nature of this protein remains to be elucidated. A direct and positive role of polyamines in the induction of encystation has been ruled out (Srivastava and Shukla, 1982). The ability of a number of structurally unrelated chemicals to bring about encystation suggests that an ideal encystation agent does not possess any specific molecular requirement. Aliphatic or aromatic nature or hydrophilicity or hydrophobicity do not appear to be the determinant characteristics. Higher concentration of magnesium may itself alter the membrane characteristics of amoeba resulting in encystation suggesting that even the presence of organic effectors is not absolutely essential. The diverse agents shown to induce encystation do however interact with

specific receptors on cell surface, induce conformational alterations and activate adenylate cyclase (Srivastava and Shukla, 1983a,b).

Excystation

Excystation represents the reverse of what all we have discussed hitherto in relation to encystation. Kaushal (1976) and Kaushal and Shukla (1975, 1976, 1977a,b,c, 1978a,b,c) have standardized the conditions of growth of *A. culbertsoni* in a simple axenic medium the encystation of the trophozoites and the subsequent exystation of the cysts to viable trophozoites. The nature of the excystation effectors has also been elucidated.

Morphological changes

Gross changes noticed when a cyst hatches are shrinking of cytoplasm from the wall, movement of the amoeba within the wall occupation of one area of the inner wall by pseudopoidal movement, appearance of an opening in the wall, emergence of the amoeba from the cyst cast and in some instances total dissolution of the cast. (McConnachi, 1969; Singh and Das, 1970). The stages have been classified as maturation, activation, pre-emergent phase and eventual emergence (Matter and Byers, 1971). Scanning electron microscopy reveals the presence of ostioles on the surface and the formation of holes (Chamber and Thompson, 1972).

Excystation factors

Axenically prepared cysts of *A. culbertsoni* readily excysted in the presence of heat-stable factors present in aqueous extracts of *Escherichia coli, Klebsiella aerogenes, Staphylococcus aureus, Sarcina lutea, Bacillus subtilis, Bacillus megaterium, Aspergillus niger, Chaetomuim globosum, Myrothecuim verrucaria, Trichodermaviridi.* Peptones and protein hydrolysates also induce excystation. No linear relationship is observed between the amount of the agent and the degree of excystation. Continuous presence of excystation factor is essential and the removal of the agent stops further excystation. Presumably no triggering mechanism is involved (Kaushal and Shukla, 1977a) in contrast to encystation where activation of adenylate cyclase is a prerequisite.

Detailed fractionation studies using aqueous extracts of *E. coli* indicate that the effectors of excystation are heat stable, dialysable and comprise low molecular weight substances. Most of the excystation promoting activity is associated with a fraction rich in amino acids. Glutamic acid shows the highest activity followed by serine, thereonine and alamine (Kaushal and Shukla, 1977a,b). γ-Amino butyric acid with an amino and carboxyl group and an intervening flexible chain of 3-carbon atom appears to have the optimum structural requirement for inducing excystation. These features are present in glutamic acid. Compounds which possess one or the other feature of these basic requirements promote varying degrees of excystation (Kaushal and Shukla, 1977c)

Mechanism of excystation

Mitomycin C and Actinomycin D have practically no effect on excystation whereas cycloheximide exerts a 100 % inhibition. Dinitrophenol and sodium arsenite also block

encystation induced by glutamic acid. The data presented in figure 3 reveal that there is significant increase of protein, marginal increase of RNA but very little DNA synthesis during excystation. There is negligible incorporation of thymidine-[^{3}H]- into DNA but a rapid incorporation of uracil-2-[^{14}C]- into RNA and incorporation of chorella protein hydrolysole-U-[^{14}C]- into proteins take place. Studies with nucleic acid and protein synthetic inhibitors suggest the involvement of the process of translation but not transcription in excystation (Kaushal and Shukla, 1978a,b).

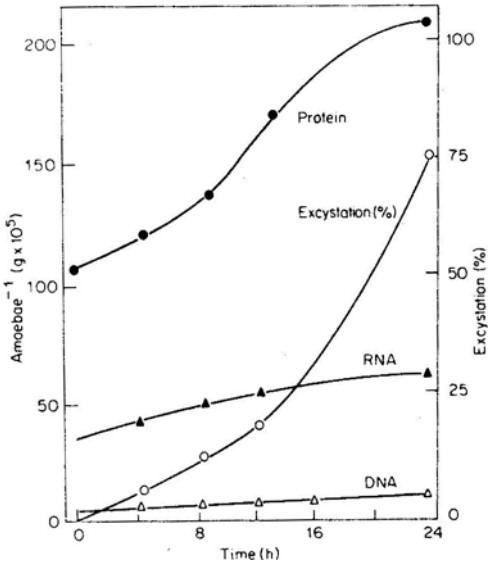

Figure 3. Macromolecular content and excystation of *A. culbertsoni.*

Release of depolymerizing enzymes

Autoclaved cysts of *A. culbertsoni* provide carbon sources for the growth of an *Aeternaria* sp. The fungus degrades the cysts, secretes into the medium protease, cellulase and chitinase and digests the cysts (Verma *et al.,* 1974). Excystation of *A. culbertsoni* in a liquid medium containing peptone and sodium chloride or aqueous extracts of *E. coli* is accompanied by secretion of two proteases, cellulase and chitinase (table 2). The proteases are secreted during the early phase whereas the disappearance of empty cysts or casts begins 48 h later coinciding with the release of cellulase and chitinase. Formation of an opening on the cyst wall is an essential step in excystation and Neal (1960) has suggested that the escape hole is produced by the action of enzymes located or synthesized in the cell membrane. The early secretion of proteases by cysts of *A. culbertsoni* suggests that they may have an important role to play in excystation. Cellulase and chitinase, on the other hand, are secreted only after excystation is completed and may be important in the subsequent degradation of empty cyst walls or the casts (Kaushal and Shukla, 1976). The alkaline protease released during excystation has been purified 100 fold by Sephadex G-100 chromatography and shown to have a molecular weight of 21400, pH optimum of 8-9 and a temperature optimum around

Table 2. Excystation of *A. culbertsoni* in presence of aqueous extract of *E. coli* and release of enzymes.

	24 h	48 h	72 h	96 h
Excystation (%)	66	73	74	74
Degradation of empty cysts (%)	Nil	45	81	100
Cellulase activity in medium	Nil	Nil	307	405
Chitinase activity in medium	Nil	Nil	420	970
Protease I (pH 5) activity in medium	450	1500	1040	Nil
Protease II (pH 9.5) in medium	735	4095	5120	3720
Cellulase activity	μg glucose liberated in 60 min by 100 ml			
Chitinase activity	μg N-acetyl glucosanine 60 min by 100 ml			
Protease I and II activity	μg tyrosine liberated in 60 min by 100 ml			

From Kaushal and Shukla, (1976).

55°C. The enzyme is inhibited by phenyl methyl-sulphonyl fluoride and appears to be a serine protease (Kaushal and Shukla, 1978c). In an extended study using cellulose labelled with glucose-U-[^{14}C]- as substrate, cellulase activity could be detected in the early phase of excystation. Dormant cysts do not possess protease, cellulase or chitinase activities. Protease I and protease II appear in the excysting cell in 24 h, reach the maxima on the 2nd day and decline thereafter. Cellulase and chitinase also behave in a similar manner. In the culture medium the appearance of enzymes shows a slightly different pattern suggesting that all the depolymerizing enzymes are associated with the cells or are synthesized and then only partially released. Incubation of the cysts with a mixture of the three depolymerizing enzymes does not bring about excystation but causes many morphological alterations (Kaushal and Shukla, 1978a,b).

Differentiation of parasitic amoebae

Information on the morphology, nutrition and general biochemistry of *Entamoeba histolytica* is far from adequate to be of any critical use in the design of drugs or other therapeutic measures (Mitra, 1975). *E. histolytica* can be maintained in the laboratory along with associate intestinal microflora. Virulent cultures have been used to simulate human amoebiasis in experimental animals. Intracaecal inoculation of such cultures into young rats or cats or into the liver of hamsters have been employed to give models for intestinal amoebiasis and amoebic hepatitis respectively. The introduction of the technique of axenization by Diamond (1968a,b) has also been not of much help in achieving infection by bacteria-free amoebae or in producing cysts.

Cyst formation can be readily demonstrated in *E. histolytics* cultures maintained with associate bacteria (Balamuth, 1951; Balamuth and Weiboldt, 1951; Dobell and Neal, 1952; Diamond, 1968a,b). However, the results obtained with axenic cultures have not been unequivocal. One of the few leads we have today in a positive direction in this regard is the outcome of the painstaking work of Mitra (1975) who devised a technique of prior exposure of axenic cultures to cholera toxin in order to prime the triggering action of epinephrine. Axenically grown *E. histolytica* does not bind epinephrine unless it is exposed to cholera toxin. Population of amoebae exposed to a combination of

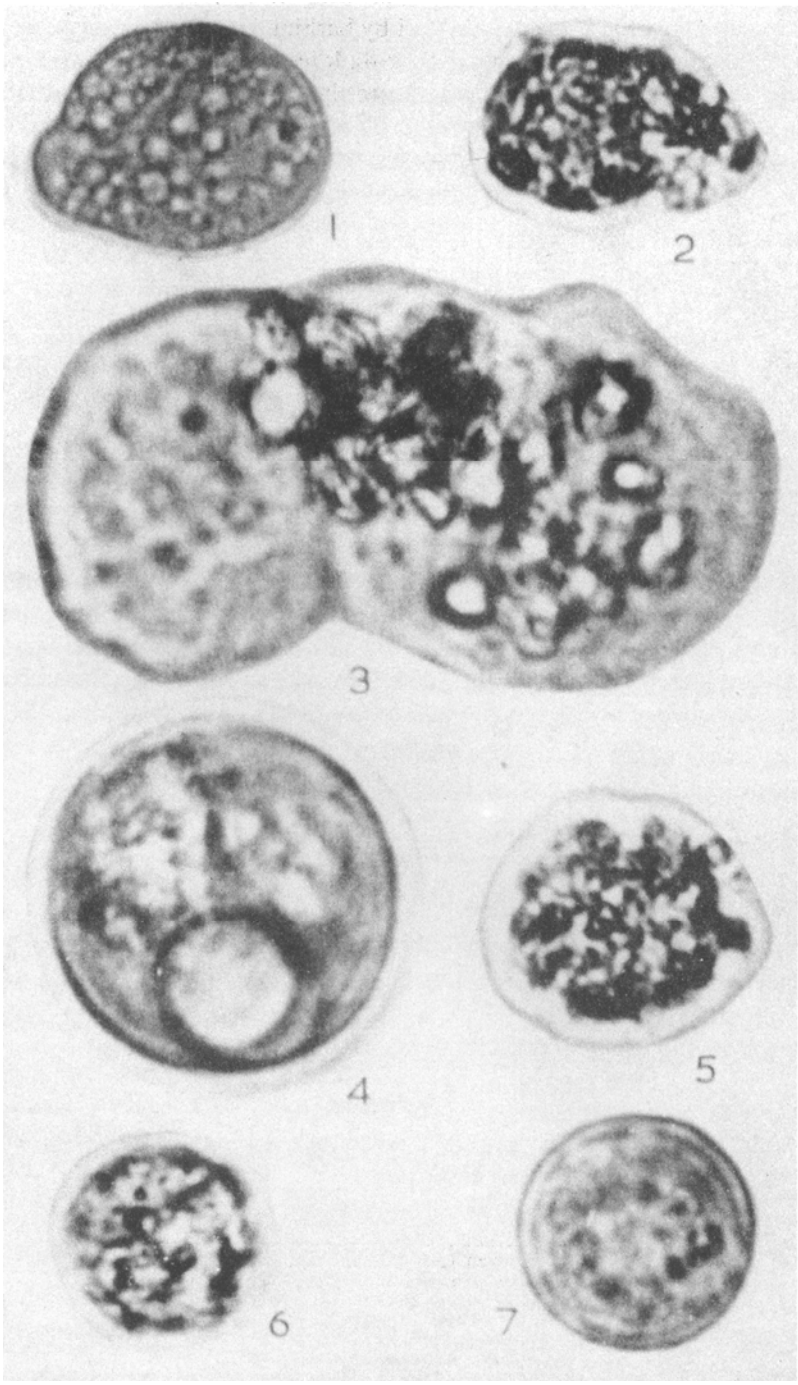

Figure 4. Morphological changes in axenically grown *E. histolytica* exposed to toxin and epinephrine.

treatment schedules which include the allowing of amoebae to ingest starch particles and exposure to cholera toxin followed by bathing in epinephrine exhibit a variety of interesting morpholigical changes (figure 4). It has not been possible so far to ascertain whether the round bodies thus formed are the polynucleated mature cysts passed by human carriers and which on ingestion by susceptible hosts excyst in the colon and multiply to give rise to amoebic dysentery (Mitra and Krishna Murti, 1978).

The questions that remain to be answered in regard to *E. histolytics* are:

(i) Is there a loss of surface receptors of biogenic amines or other encystation effectors when *E. histolytica* trophozoites are axenized?
(ii) What are the factors present in the mileu of the human gut and its commensal microflora which could be acting as encystation and excystation effectors?
(iii) Does the loss of virulence and invasiveness associated with axenization also mean the loss of the ability of the cells to receive the chemical signals of encystation effectors?

Molecular biology of differentiation of amoeba

The cascade of events occurring in the differentiation of *A. culbertsoni* is summarised in figure 5. The inhibitory effect of Actinomycin D on encystation under different conditions suggests that the control is exerted at the transcriptional level. Catabolic repression by glucose, acids and in general by carbon and nitrogen could be taken as suggestive evidence of the blocking of transcription by repressors synthesized as the

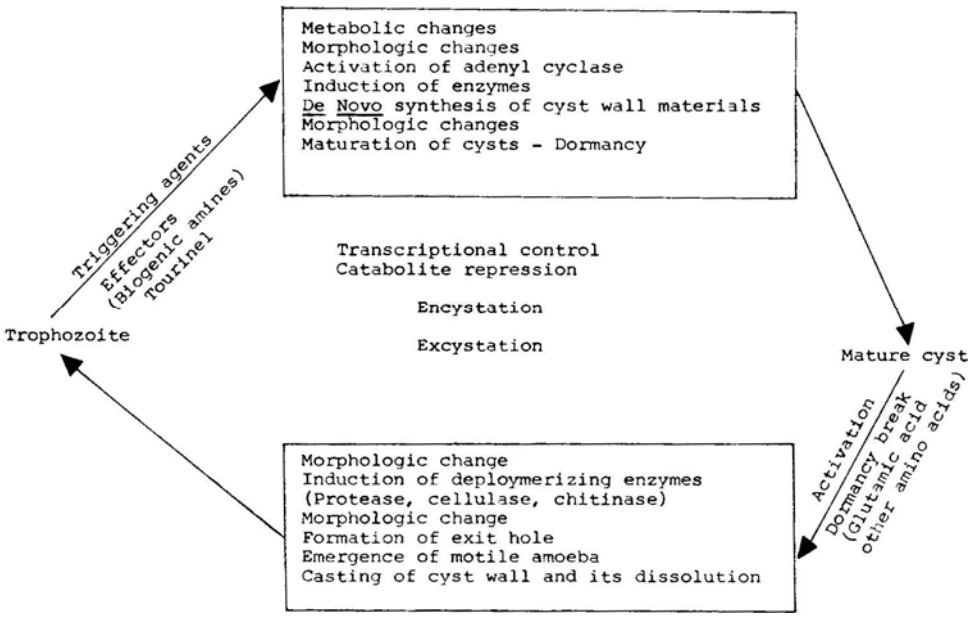

Figure 5. Cascade of events in differentiation of *A. culbertsoni.*

trophozoites age. The inhibition imposed is unmasked by the effectors which trigger encystation through the intermediately messenger cyclic AMP. Phosphorylation of cytosol proteins can be assumed to occur as a logical sequel leading to conformational alterations and consequently to new functional activity. Reserve food materials like glycogen and lipids are hydrolysed. Glucose is used directly for building cellulose and after amination changed to glycoproteins. The enzymes required for the biosynthesis of the building materials are induced by derepression. Polyamines known to regulate derepression in many prokaryote and eukaryote cells do not seem to be involved in the differentiation of *A. culbertsoni.* Morphological changes facilitate the withdrawal of pseudopodia and the layering of the outer walls and their maturation into the impermeable coat.

The excystation of cysts bears a gross resemblance to the germination of bacterial spores or vegetable seeds in the matter of the requirement of an activation of "awakening" phase when the dormant amoeba is activated from its sluggish state to one of rapid movement. Glutamic acid and other amino acids function as effectors for this activation by an as yet unelucidated mechanism. Activation and dormancy break are inhibited by cycloheximide suggesting that the control is exerted at the translational level. Depolymerizing enzymes are induced. The proteases attack peptide bonds on the structural constituents causing the appearance of exit passages or escape holes. Cellulase and chitinase also help in this process but much more in solubilizing the case or the empty cyst wall and releasing nutrients for the growth of amoebae.

Divalent cations, particularly, magnesium and possibly calcium appear to be essential for the biochemical reactions in both encystation and excystation and the fluxes of these ions help presumably in maintaining osmotic balance and in the attainment of the final architecture of the cysts in encystation as well as in the loosening of the cyst wall during excystation.

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