ENCYSTMENT OF AMOEBAE : AN EXAMPLE OF SINGLE CELL DIFFERENTIATION*

C. R. KRISHNA MURTI

Central Drug Research Institute, Lucknow

Encystment and Amoebiasis

MANY protozoa undergo encystation—a process by which motile amoebae lose their characteristic pseudopoidal movement and become covered by a protective sheath made up of a double layered wall. The resulting cysts are immotile and metabolically inert but are inherently capable of resuming trophic or vegetative existence by shedding the cyst coat when conditions are favourable for excystment. Although encystation is only a defensive posture adopted by the cell to encounter the stress imposed by a hostile environment, transformation of the trophic amoeba to the cystic form and of the viable cyst back to the trophozoite involves cellular differentiation[1-4].

The elucidation of this process of differentiation at the molecular level besides its intrinsic academic interest is of vital importance in the successful eradication of amoebiasis. *Entamoeba histolytica* is the causative agent incriminated in amoebic dysentery, amoebic colitis and amoebic appendicitis. Attempts to demonstrate an association of the pathogenicity of *E. histolytica* with toxins or invasive enzymes elaborated by it have so far been futile and as such there is no rational experimental basis today for initiating a programme of passive or active immunization against the disease[5]. Coexisting with the commensal bacterial flora of the human gut, *E. histolytica* multplies vigorously and undergoes encystment and excystment. Ulceration in the gut wall and abscess formation in the liver are brought about by the multiplication and erosive actior of the trophic form of the pathogen. The transmission of parasitic *E. histolytica* from one host to another is, however, mediated by the cysts. Human carriers of E. *histolytica* may not exhibit overt symptoms of the disease and yet can pass out viable cysts of the parasite. Multiplication of the trophic form can be arrested by treatment with enterovioform or nitroimidazole derivatives which act in the bowel lumen, antibiotics which eliminate the associate bacteria and render the amoeba innocuous and chloroquine which brings about the remission of liver abscess. The cysts, on the other hand, are impermeable to these agents. The urgent need for the discovery of potent cysticidal agents has, therefore, been repeatedly emphasized by the Expert Study Group of WHO[6]. A rational design of cysticidal agents can be attempted only after the chemical composition of the cyst wall and the molecular biology of encystment are described in precise terms.

In an extensive review on the morphology, formation and development of cysts of Entamoeba, McConnachie[7] has drawn attention to the inadequacies in the currently available information on the biochemistry of encystment. The environment of the human gut and its native microflora appear to be essential for *E. histolytica* to encyst[8]. The pathogen can, however, form cysts outside the gut in monoaxenic cultures. Yet, attempts to produce *in vitro* cysts with axenically grown *E. histolytica* have been hitherto unsuccessful. What is the nature of the triggering agent in the human gut that signals the amoebae to encyst ? Is encystment only a defence measure for survival against a hostile environment ? Are the drastic metabolic changes that occur concurrently with the morphological transformation during encystment, controlled by the genome of the amoeba ? As the molecular events enacted by *E. histolytica* during encystment have not been completely mapped and quantitated, the above questions have remained unanswered.

In contrast to the behaviour of *E. histolytica*, free living pathogenic soil amoebae such as *Acanthamoeba* or *Hartmannella* can be induced to undergo encystation under strictly axenic conditions. Such systems, obviously, provide a useful model for the study of the mechanism of encystment and have been gainfully employed in some recent investigations. The biochemistry of the encystment of *Acanthamoeba* sp. has been discussed in some detail

---

* Communication No. 1675 from the Central Drug Research Institute, Lucknow. based partly on a talk given to the participants of the Summer Institute of Advanced Biology at the Department of Biological Sciences, Madurai University, Madurai, on June 28, 1971.

by Neff and Neff[9]. Emphasising the striking resemblance of encystment to certain phases of embryonic differentiation, they conclude that during encystation amoebae do not divide but only go through a period of induction followed by the morphological changes leading ultimately to the formation of the cyst. In the process, however, new types of macromolecules appear, the emergence of which is blocked by inhibitors of the synthesis of RNA and protein.

Encystment of Axenically Grown *H. culbertsoni*

As part of a research project on the chemotherapy of amoebiasis, some aspects of the encystment of a free living soil amoeba identified by Singh and Das[10] as *Hartmannella culbertsoni* have been investigated by the author and his associates. The aim of this brief review is to highlight some of the pertinent observations of this study.

H. culbertsoni can be grown axenically in a proteose-peptone-tryptone medium. On transferring six days old cultures of *H. culbertsoni* to replacement media no encystment is noticed. Nor does the incorporation in the growth medium of mitomycin C, actinomycin D, cycloheximide, *p*-fluorophenylalanine or azaadenine at later phases of growth lead to encystation. However, on incubating six days old amoebae on non-nutrient agar plates containing 10 mM NaCl, 15 mM $MgCl_2$ and 20 mM taurine ($NH_2.CH_2.CH_2.SO_3$) 80–90% of the trophozoites are transformed into viable double walled cysts in the course of 72–96 hrs. The idea of using taurine, betaine, adrenaline or noradrenaline, all derivatives of amino-acids, suggested itself from the following earlier observations: (*i*) free living amoebae undergo encystation in the presence of bacteria[11] and (*ii*) the colon where *E. histolytica* normally encysts is rich in amino-acid degradation products arising out of bacterial metabolism. Fortunately this surmise turned out to be correct and after a number of trials using adrenaline, noradrenaline, betaine, taurine or creatinine, the above-mentioned combination of Mg^{2+} and taurine in saline has been found to be optimal for the *in vitro* encystment of *H. culbertsoni*[12].

Periodic microscopic examination of the amoebae exposed to the above encysting medium reveals typical morphological changes such as the withdrawal of the pseudopodia, rounding up of the amoebae, shrinkage in cell size and the final building up of a double layered cyst wall. When transferred to the growth medium, viable cysts obtained by this procedure can excyst. The trophozoites formed thus could once again be transformed into viable cysts by exposing them to the combined action of Mg^{2+} and taurine and the cycle of differentiation repeated as many times as one wished. Since *H. culbertsoni* undergoes encystation readily in the presence of bacteria[11], every possible care has to be taken to exclude bacteria in the *in vitro* induction of encystation of the amoebae by Mg^{2+} and taurine. Mitomycin C, actinomycin D and cycloheximide are all uniformly effective in appropriate concentration in arresting encystment. It is evident, therefore, that information transfer is involved in the induction of encystation and if a break has to be applied to the underlying process of differentiation it must be at one of the steps of genetic expression[12].

The exposure to Mg^{2+} and taurine leads to changes in the internal milieu of the amoebae directed by a regulatory mechanism under genetic control. Formation of the cyst wall is the ultimate morphological manifestation of the totality of such changes. It is not necessary that the amoebae have to be continuously exposed to Mg^{2+} and taurine to be "turned on" for differentiation. Encounter with these two agents for a period of six hours initially is all that is required. After this, when they are transferred to non-nutrient agar containing only 10 mM NaCl, the same degree of encystment ensues in 72–96 hrs. as given by keeping the amoeba throughout the period in the encysting medium. The action of Mg^{2+} appears to be related to osmotic effects resulting in the leakage of cellular constituents and is somewhat analogous to what has been reported to occur in the slime mould differentiating under the action of this cation. The exit of essential metabolites from the cytoplasm can presumably lead to dormancy. Alternatively, Mg^{2+} may be playing a role in specifically activating or stabilizing the ribosomes on which the enzymes required for the manufacture of cyst wall constituents are assembled as a result of translation. The mechanism of triggering effected by Mg^{2+} and taurine remains to be studied.

Role of Cyclic AMP in Encystment

Acrasin, a factor required for the aggregation of the slime mould *Dictyostetium discodium*, has now been identified as cyclic AMP[14-21]. For the slime mould to differentiate, aggregation and formation of clumps appear to be an essential

preliminary step. Such aggregation is stimulated by c-AMP and, furthermore, strains or species of amoebae which excrete phosphodiesterase into the medium do not aggregate as readily as those which do not excrete the enzyme. Thus, the relative concentrations of phosphodiesterase and cyclic AMP in the periphery of the amoebae seem to exert a regulatory influence by preventing or facilitating aggregation[22-23]. Although other nucleotides have the same effect on the morphogenesis of *D. discosdium*, c-AMP is perhaps the natural activator. It is relevant in this context to mention that c-AMP also influences the morphological transformation of mammalian cells such as normal fibroblasts[24] or Chinese hamster cells[25]. The action again of c-AMP in regulating enzyme induction in bacteria is well documented[25-31]. Already established as the chemical mediator of hormonal action in both animals[32] and plants[33,34], cyclic AMP, among a multiplicity of functions, seems to be involved in the metabolic regulation and differentiation of unicellular organisms as well.

By experiments designed to explore whether the Mg^{2+} and taurine induced encystation of *H. culbertsoni* is mediated by c-AMP, Raizada and Krishna Murti have been able to show that c-AMP mimicks the role of Mg^{2+} and taurine in the transformation of trophozoites to cysts. Furthermore, trophozoites exposed to Mg^{2+} and taurine in the non-nutrient medium synthesize three to four times the amount of c-AMP as do the control trophozoites. While the encystation inducing action of c-AMP is shared by theophylline—an inhibitor of phosphodiesterase, 5′ or 3′ AMP do not exhibit this property but ADP stimulates encystation to some extent. Amoebae exposed to Mg^{2+} and taurine as well as the medium in which they are encysting show decreased levels of phosphodiesterase activity. The above evidence leads to the inescapable conclusion that c-AMP is perhaps the agent that triggers the molecular events that lead to the encystation of *H. culbertsoni*[35].

Since cyclic AMP mediated cellular reactions require preactivation of a membrane bound adenylcyclase, it is of interest to know whether taurine is picked up by the membranes of *H. culbertsoni*. By measuring the incorporation of taurine-S^{35} on the membrane system of trophozoites exposed to the encysting medium, one finds that Mg^{2+} ions do indeed stimulate the specific binding of taurine to the membranes[36]. On the basis of this observation it may be inferred that after binding of taurine to the membranes, particulate adenyl cyclase is activated leading in its turn to a synthesis of cyclic AMP. The nature of the specific macromolecule to which c-AMP is bound remains to be uncovered. Modulation of a protein, especially an enzyme with a sub-unit structure to an altered conformation, is now accepted as the *modus operandi* of c-AMP.

Macromolecular Changes During Encystment

The morphological changes leading to the differentiation of trophozoites to cysts are accompanied by alterations in the chemical composition of the cellular milieu, enzyme profile and serological character of the trophozoites. The most dramatic change that takes place during the differentiation is the switching over of the metabolism of the trophozoites from aerobiosis to anaerobiosis ending up in a cellular form almost inert metabolically. Soluble carbohydrates, phosphates, RNA, DNA and proteins decrease in content whereas the concentration per unit mass of complex carbohydrates such as cellulose and mucopolysaccharides increases. The activity of dehydrogenases mediating the aerobic degradation of glucose carbon for energy production gradually decreases. In contrast, enzymes required for the building up of polymer constituents or cyst wall such as mucopolysaccharides and cellulose increase in activity. Synthesis of cellulose as measured by the incorporation of glucose-U-C^{14} into a β-linked polymer of glucose (as evident from its susceptibility to cellulase) is stimulated nearly thirty-fold during the first twenty-four hours of exposure of the trophozoites to the encystation medium. The induction of these enzymes is also blocked by actinomycin D and cycloheximide suggesting that their synthesis involves transcription and translation[37].

Concurrently with the above biochemical changes, the encysting cells acquire new serological characters. The precipitin type of antigens specific to cysts are visualized on Ouchterlony plates only after their exposure to trypsin whereas the trophic antigens that readily show precipitin lines with their homologous antiserum are inactivated by trypsin. Cystic antigens fix guinea-pig complement both specifically (in the presence of homologous anti-sera) and non-specifically. The specific complement fixation by cystic antigens is not affected by trypsin whereas the specific fixation of guinea-pig complement by trophic antigens

is abolished by trypsin. Furthermore, the non-specific fixation of complement, a property exhibited only by cysts, is inhibited by cellulase[38].

Outlook for the Future

The single cell differentiation bringing about encystment and excystment of *Hartmannella* is diagrammatically represented in Fig. 1.

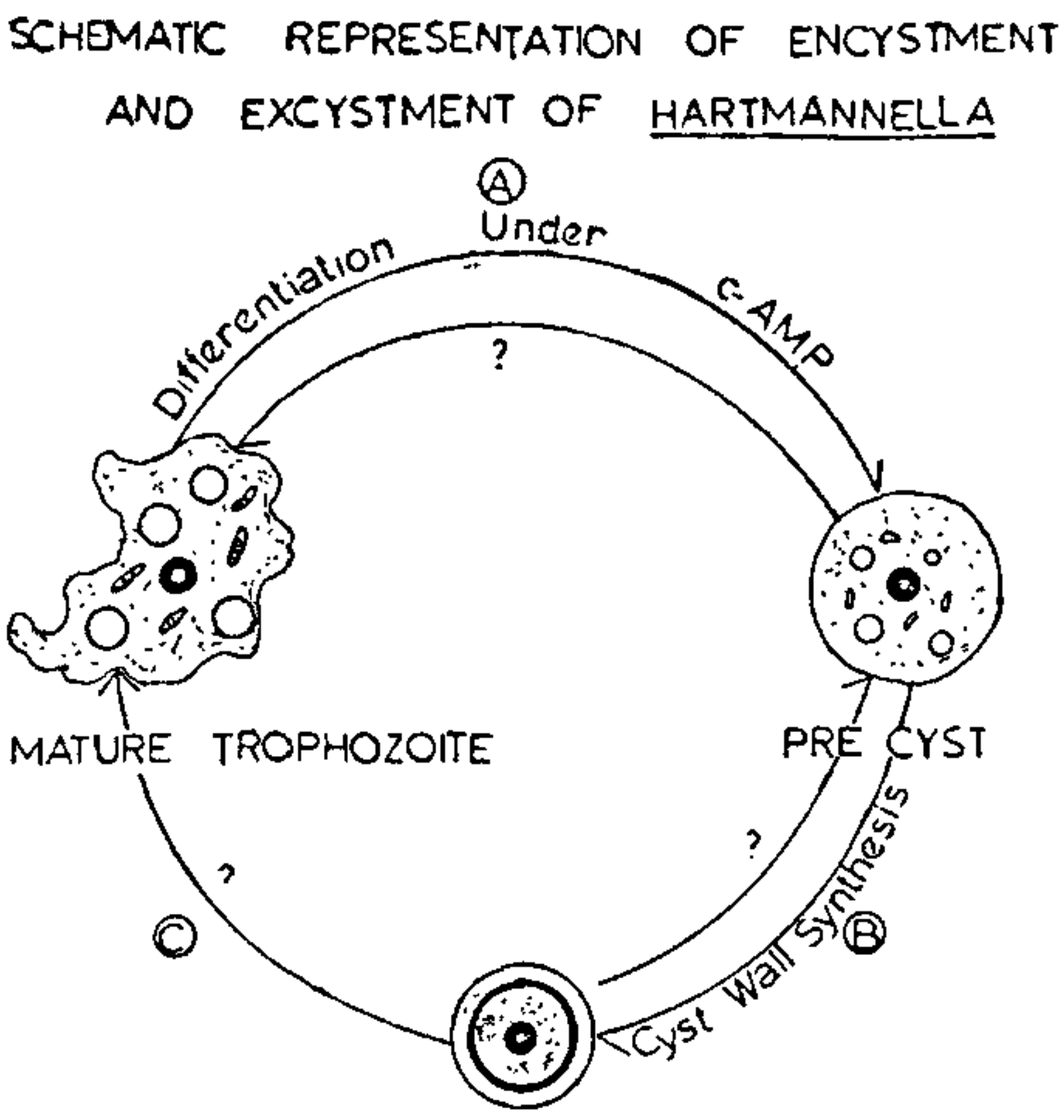

FIG. 1

Events during Step A: 1. Binding of taurine to membrane and activation of adenyl cyclase and increase in the concentration of intracellular *c*-AMP. 2. Massive degradation of reserve materials and shrinkage of mitochondria. 3. Shift from aerobiosis to anaerobiosis.

Events during Step B: 1. Induction of enzymes needed for the synthesis of cellulose and mucopolysaccharide. 2. Disappearance of mitochondrial activity. 3. Attainment of dormancy.

Events during Step C: 1. Imbibition of water. 2. Activation of trophozoites. 3. Shedding of outer cyst coat. 4. Emergence of active trophozoite.

On morphological evidence, it can be assumed that the events from A to B through precyst stage are reversible. Reversibility of the biochemical events has, however, not been experimentally established.

The induction stage in the encystment of *H. culbertsoni* involves the shutting off of aerobic metabolism and a loss in respiratory activity with attendant destruction or shrinkage in the size of the mitochondrial apparatus. Entamoeba, in contrast, is a typical anaerobe and its very survival depends upon the maintenance of strict anaerobic conditions. Though environmental factors, particularly nutrition and redox potentials, have been considered to be important in bringing about the encystation of Entamoeba[39-41], metabolic changes during encystment have not been mapped in any detail. If encystation is indeed preceded by cessation of growth, starvation and massive breakdown of reserve food materials, the attendant degradative changes may be expected to be favoured by anaerobic rather than aerobic conditions. It may well be that for Entamoeba to encyst only an induction phase is required for the elaboration of the enzymes mediating cyst wall synthesis and no substantial change in the energetics of the cell. Cysts of *Entamoeba* have been reported to survive even under aerobic conditions depending upon the amount of glycogen and chromatoid body available in the cytoplasm. This would imply that in the encystment of *E. histolytica* there may be a switchover from anaerobiosis to aerobiosis, the reverse of what is occurring in encysting aerobic *Hartmannella* Nonetheless, with the knowledge available of such induction in *H. culbertsoni,* it should be relatively easy now to demonstrate the synthesis of mucopolysaccharides and cellulose in encysting *E. histolytica.*

Axenically grown cultures of pathogenic strains of *E. histolytica* have not so far been transformed *in vitro* into viable cysts. Axenically grown *E. histolytica* are also relatively non-pathogenic to experimental animals. In contrast, monoaxenically or diaxenically grown cultures of *E. histolytica* are very virulent and also undergo encystation both *in vivo* and *in vitro.* The significance of this difference in behaviour between axenically grown and non-axenically grown *E. histolytica* remains to be elucidated. Avirulent laboratory cultures of *E. histolytica* readily become virulent on animal passage or on repeated subculturing in media rich in cholesterol[42,43]. A study of the cholesterol metabolism of *E. histolytica* in axenic cultures may help in understanding the change of virulence to avirulence. Detailed investigations on the synthesis and degradation of macromolecules by the sequential creation and destruction of cyclic AMP or other regulatory molecules have to be conducted. The indentification of factors secreted by bacteria that apparently bring about the encystation of *E. histolytica* in the human gut would appear to be yet another fruitful line of work.

The evidence adduced so far on the biochemistry of encystment using free living amoeba as model suggests that the synthesis of cellulose synthetase or mucopolysaccharide

synthetase are coded by new species of *m*RNA's. Almost a similar situation is encountered in the sporulation of micro-organisms. The imposition of dormancy over a vegetative phase characteristic of amoebic encystment or bacterial sporulation is perhaps under the control of a unitary regulatory mechanism involving cyclic nucleotides. In the light of this, amoebic excystment, or for that matter, germination of a bacterial spore, may be controlled by the opposite counterpart of the regulatory mechanism operating encystment or sporulation. The molecular biology of encystment and excystment is thus two sides of the same coin, namely, a process of a single cell differentiation.

1. Trager, W., *J. Protozool.*, 1963, **14**, 114.
2. Willmer, E. N., *Symp Soc. exptl. Biol.*, 1963, **17**, 215.
3. Sussman, M., *Ann. Rev. Microbiol.*, 1965, **19**, 59.
4. Wright, B. E., *Science*, 1966, **153**, 83.
5. Brown, H. W. and Belding, D. L., In *Basic Clinical Parasitology*, Second Edition, Appleton Century Crofts, New York, 1964, pp. **17**, 34.
6. Anon, *WHO Technical Report Series*, 1969, p. 421.
7. McConnachie, E. W. *Parasitol.*, 1969, **59**, 41.
8. Balmuth, W., *J. Parasitol.*, 1962, **48**, 101.
9. Neff, R. J. and Neff, R. H.. *Symp. Soc. Exp. Biol.*, 1969, **23**, 51.
10. Singh, P. N. and Das, S. R., *Phil. Trans. Roy. Soc., B*, 1970, **259 B**, 435.
11. —, Saxena, U. and Iyer, S. S., *Ind. J. expl. Biol.*, 1965, **3**, 110.
12. Raizada, M. K. and Krishna Murti, C. R., *J. Protozool.*, 1971, **18**, 115.
13. Yea Yuan Chi and Francis, D., *J. Cell. Physiol.*, 1971, **77**, 169.
14. Konijn, R. M., Vande-Meeme, J. G. C., Bonner, J. T. and Barkley, D. S , *Proc. Nat. Acad. Sci. U.S.*, 1967, **58**, 452.
15. Konijn, T. M., Barkley, D. S., Chang, Y. Y. and Bonner, J. T., *Ann. Naturalist.*, 1958, **102**, 225.
16. —, Vande-Meeme, J. G. C., Chang, Y. Y., Barkley, D. S. and Bonner, J. T., *J. Bact.*, 1969, **99**, 510.
17. Krichevsky, M. I., Lowe, L. L. and Chassy, B. M., *J. gen. Microbiol.*, 1969, **57**, 383.
18. Barkley, D. S., *Science*, 1969, **165**, 1133.
19. Hall, E. M., Konijn, J. M. Mason, J. W., O'keefe, G. and Wolfe, P. B., *Dev. Biol.*, 1969, **20**, 72.
20. Chassey, B. M., Love, L. L. and Krichevsky, M. I., *Proc. Nat. Acad. Sci. U.S*, 1969, **64**, 296.
21. Bonner, J. T., *Ibid.*, 1970, **65**, 110.
22. Riedel, V. and Gerisch, G., *Biochem. Biophys. Res. Communs.*, 1971, **42**, 119.
23. Murray, A. W., Spiszman, M. and Atkingron, D. E., *Science*, 1971, **171**, 496.
24. Johnson, G. S., Friedman, R. M. and Pastan, I., *Proc. Nat. Acad. Sci. U.S.*, 1971, **68**, 425.
25. Hsie, A. W. and Pack, J. T., *Ibid.*, 1971, **68**, 358.
26. Goldenbaum, P. E. and Dobyogosz, W. J., *Biochem. Biophys. Res Commn.*, 196 , **33**, 828.
27. Perlman, R. L. and Pastan, I., *Ibid.*, 1968. **30**, 656,
28. — and —, *J. biol. Chem.*, 1968, **243**, 5420.
29. Pastan, I. and Perlman. R. L , *Proc. Nat. Acad. Sci. U.S.*, 1968, **61**, 1336.
30. Perlman, R. L., deCrombrugghe, B. and Pastan, I., *Nature*, 1969, **223**, 1810.
31. deCrombrugghe, B., Perlman, R. L., Varmus, H. E. and Pastan, I., *J. Biol. Chem.*, 1969, **244**, 5829.
32. Sutherland, E. W. and Rall, T. W., *Pharmacol. Rev.*, 1960, **12**, 265.
33. Follard, C. J., *Biochim. biophys. Acta*, 1970, **201**, 511.
34. Azhar, S. and Krishna Murti, C. R., *Biochem. Biophys Res. Communs.*, 1971, **43**, 58.
35. Raizada, M. K. and Krishna Murti, C. R., Submitted to *J. Cell. Biol.*
36. — and —, Unpublished Work.
37. — and —, Submitted to *Exp. Cell Res.*
38. —, Saxena, K. C. and Krishna Murti, C. R., *J. Protozool*, (In press).
39. Balamuth, W., *J. Infect. Dis* , 1951, **88**, 230.
40. Chang, S. L., *Parasitol.*, 1946, **37**, 101.
41. Everritt, M., *J. Parasit* , 1950, **36**, 586.
42. Sharma, R. *Trons. Roy. Soc. Lop. Med. Hyg.*, 1959, **53**, 278.
43. Singh, B. N., Srivastava, R. V. N. and Dutta, G. P., *Ind. J. exp. Biol.*, 1971. **9**, 21.