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INHIBITION OF POLYAMINE METABOLISM INDUCES AMOEBIC ENCYSTATION

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ACANTHAMOEBA CULBERTSONI, the pathogenic free-living amoeba causes fatal meningoencephalitis in experimental animals as well as in man[1,2]. This organism has served as a model for differentiation of a unicellular eukaryotic cell[1,3]. This amoeba encysts readily in non-nutrient media containing magnesium chloride in combination with taurine[4], biogenic amines[5], inorganic ions[2] and other organic effectors[6,7]. An intermediary role of cAMP in encystation of this amoeba seems likely[3] and the system is also regulated by catabolite repression[8].

Aliphatic polyamines viz putrescine, spermidine and spermine are ubiquitously distributed in organisms[9,10], and regulate the biosynthesis of RNA/DNA, cell growth, differentiation, and stability of macromolecules and cellular organalles. Selective inhibition of polyamine metabolism of protozoal parasites has been identified as an alternative target for their chemotherapy. Polyamine metabolism is closely regulated during normal growth, differentiation, action of some hormones and altered regulation of polyamine biosynthesis has been observed during neoplastic growth[9,10]. Spermidine and putrescine have been identified as the major aliphatic amines in *A. culbertsoni*[11,12]. The present paper reports the changes in polyamine levels during encystation of this amoeba and the effect of certain inhibitors of polyamine metabolism on amoebic encystment.

An axenic culture of *A. culbertsoni* was grown[13] in a medium containing 2% peptone, 0.5% sodium chloride, 1 mg/100 ml thiamine and 0.5 μg/100 ml cyanocobalamine, pH adjusted[14] to 6.9–7.0. The required volume of the medium was distributed in one litre Erlenmeyer flasks, autoclaved at 15 lbs/in^2 pressure of steam for 20 min, and steamed after 24 hr to kill any germinated spores. Flasks were inoculated with 25 ml of 6-day-old static culture of *A. culbertsoni* having a cell density of 8×10^5 cells/ml, and incubated at 37 ± 2°C with shaking for four days. Amoebae were harvested aseptically by centrifugation (500 g, 10 min), washed and suspended in 0.9% saline. The basal encystment medium (NM) for testing the effect of different compounds contained 15 mM $MgCl_2$ and 86 mM NaCl (pH adjusted to 7.8). The medium (4.5 ml) was dispensed in 25 mm × 150 mm tubes and sterilized. Stock solutions of polyamines and inhibitors were sterilized by filtration through millipore filters (0.22 μm), and incorporated in the basal medium to the requisite concentration. Freshly harvested amoebae (0.5 ml) of desired cell density were inoculated in each tube, and the tubes incubated with shaking at 28 ± 2°C. Encystation was monitored in small aliquots removed from the tubes by counting the trophozoites, intermediate forms (single-walled cysts) and mature cysts (double-walled) using a haemocytometer. Viability of cells was examined by rapid eosine (0.125% w/v in water) staining. Eosine stained dead cells within few seconds while living ones remained unstained up to 15–20 min. All the operations during growth and encystment were conducted under aseptic conditions, and the sterility of media was checked at the end of experiment, by inoculating aliquots on nutrient agar slants.

Polyamines were characterized and quantitated by thin layer chromatography of their Dansyl derivates. Aliquots were removed at different incubation periods, cells counted using haemocytometer, sedimented by centrifugation and homogenized with 4% perchloric acid. The polyamines in the supernatant were converted to their Dansyl derivatives by the method of Fleischer and Russel[15] and separated on silica gel plates by ascending chromatography serially in following three solvents (a) benzene, cyclohexane, methanol (127:23:1.9 v/v) (b) cyclohexane, ethyl acetate (1:1 v/v) (c) cyclohexane; ethylacetate (3:2 v/v) as described earlier[16]. The spots were visualized by their fluorescence in UV light, scraped and suspended in 3 ml benzene, shaken in vortex mixer and centrifuged at 2000 rpm for 15 min. Fluorescence was measured in the supernatants using a spectrofluorimeter (Aminco

Bowman) with activation wavelength at 365 nm, and emission wavelength of 505 nm. Standard amounts of polyamines were processed similarly and the recovery of polyamines from amoebic homogenates was corrected accordingly.

Figure 1 presents the putrescine and spermidine content of *A. culbertsoni* cells during encystation in standard encystment medium containing NaCl, $MgCl_2$ and taurine. A rapid drop in the level of both polyamines occurred within the first 6 hr of incubation; putrescine dropped to about 60% while spermidine to about 30% of original levels. Further incubation resulted in progressive drop in polyamine levels. The rapid drop in polyamine levels during the early phase of encystment coincides with the initial commitment phase of encystation when cells get irreversibly committed to encystation[17].

In view of the drop in polyamine levels during encystation, the effect of two inhibitors of polyamine metabolism viz ∝-methyl-ornithine (∝MO), which inhibits ornithine decarboxylase, and methylglyoxal-bisguanyl hydrazone (MGBG), which inhibits S-adenosylmethionine decarboxylase, on promoting encystation was examined and the results are presented in table 1. The inhibitor ∝MO considerably enhanced

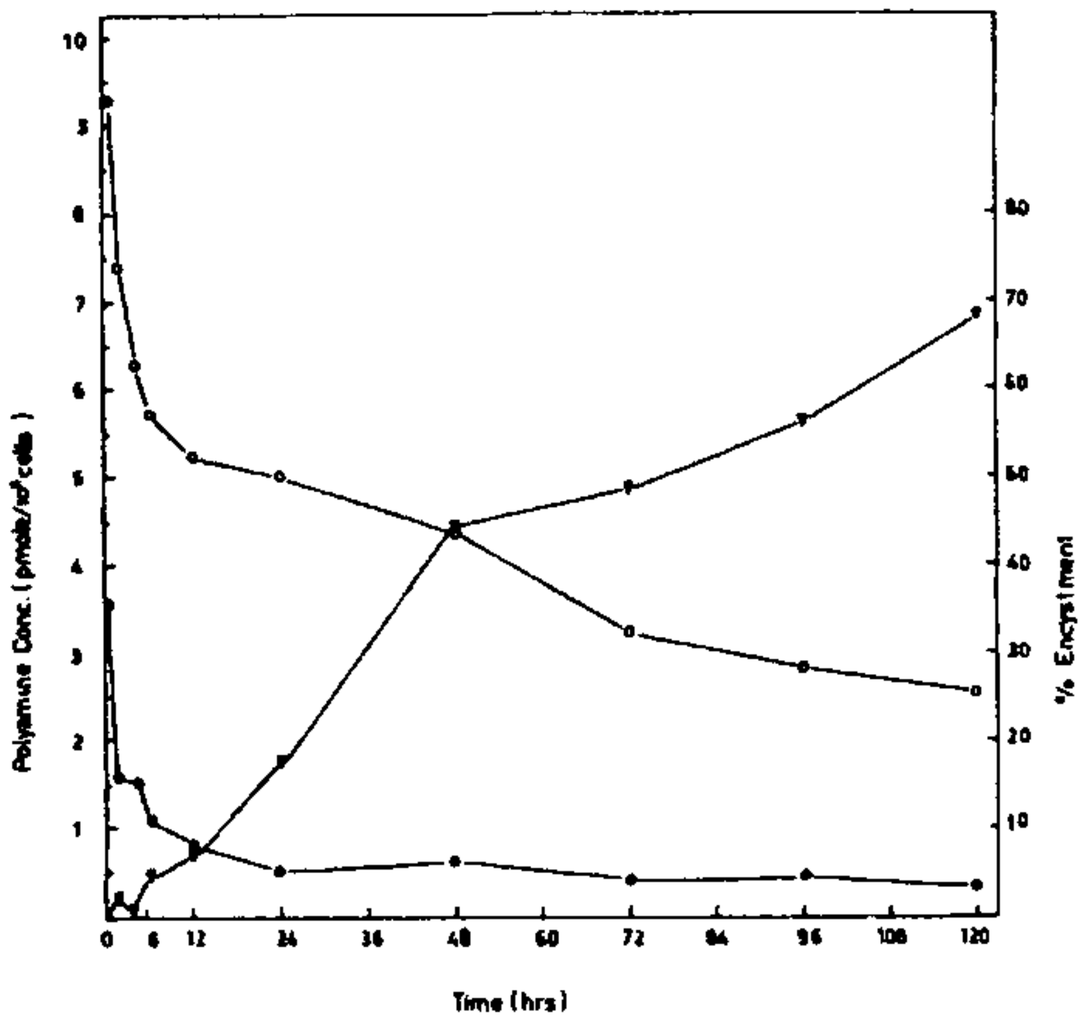

Figure 1. Changes in the level of putrescine and spermidine during encystation of *A. culbertsoni*. [o——o, Putrescine; ●——●, Spermidine; ▼——▼, Per cent encystment].

encystation, when added in NM medium at 0.1–0.5 mM concentrations. Putrescine did not promote encystation. When added with ∝-methylornithine,

Table 1 *Effect of polyamines and their inhibitors on encystment of Acanthamoeba culbertsoni*

	Media	Concentration (mM)	No. of trophozoites	No. of intermediates	No. of cysts	Total	% Encystment
			$\times 10^4$/ml				
A.	NM	—	28	9	21	58	36.20
	NM + Putrescine	0.1	16	4	9	29	31.03
	NM + ∝ Methyl-ornithine	0.1	28	11	40	79	50.63
		0.5	24	9	41	74	55.41
	NM + ∝ Methyl-ornithine + putrescine	(0.1:0.1)	28	11	28	67	41.79
		(0.1:0.5)	18	8	13	39	33.33
B.	NM	—	72	39	70	180	38.65
	NM + MGBG	0.1	60	32	109	201	54.12
		0.5	40	19	160	218	73.09
		1.0	45	20	137	203	67.67
C.	NMT	—	47	31	118	196	60.20
	N	—	60	30	35	125	28.00
	NM	—	54	31	42	127	33.07
	NM + Putrescine	1	59	33	48	140	34.29
	NM + Spermidine	1	45	21	50	116	43.10
	NM + Spermine	1	41	25	51	117	43.59

N = NaCl (86 mM), M = $MgCl_2$ (15 mM), T = Taurine (20 mM). Higher (10 mM) concentration of polyamines in the medium caused lysis of amoebae. Lysis also occurred on adding polyamines (1 or 10 mM) in NaCl medium in absence of Mg^{++}. The number of cells in set A = 1.17×10^6/ml; B = 2.90×10^6/ml; C = 2.4×10^6/ml. Data are mean of four different counts from two tubes.

putrescine counteracted the encystation-promoting activity of inhibitor; at 0.5 mM concentration putrescine almost abolished the action of ∝MO. MGBG proved more effective in promoting encystation and gave maximum encystation at 0.5 mM concentration. Polyamines themselves were not very effective in enhancing encystation. Putrescine was completely inactive while spermidine and spermine enhanced encystation to some extent at 1 mM concentration. Increase or decrease in polyamine concentration did not further enhance encystation; higher concentrations of polyamines resulted in lysis of amoebae. The presence of Mg^{++} was also required for encystation inducing effect of MGBG, and the removal of Mg^{++} lowered encystation.

The present results establish a rapid drop in polyamine levels during encystation of *A. culbertsoni*. Enhancement of encystation in the presence of ∝ MO and MGBG, suggests that inhibition of polyamine biosynthesis and decrease in polyamine levels may signal cessation of growth and activate encystation related genes. Inhibitors of DNA biosynthesis[18] and mitochondrial function[19] also trigger encystation. Polyamine metabolism may also be a target for encystment-inducing action of magnesium and some organic effectors. Putrescine, on the other hand, promotes considerable encystment of *A. culbertsoni*,[20] supporting a key regulatory role of polyamine metabolism in amoebic differentiation.

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TRANSPLACENTAL INDUCTION OF MICRONUCLEI FOLLOWING MATERNAL ADMINISTRATION OF MERCURIC CHLORIDE

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MERCURIAL compounds are known to be clastogenic, teratogenic and embryotoxic. However the foeto-maternal barrier is more effective against inorganic forms of mercury[1-3]. The present investigation was undertaken to observe the foeto-toxic effects of inorganic mercuric chloride with the help of the micronucleus test, which indicates a damage at the chromosome level. Its presence in foetal liver cells can be taken as an index of clastogenicity in the foetal tissue[4-9].

Three subtoxic doses of mercuric chloride (LD_{50} = 37 mg/kg body wt.)[10] were administered orally to an inbred laboratory strain of pregnant female rats *Rattus norvegicus* on day 7 of gestation. The doses used were 1/20th LD_{50} (1.85 mg/kg body wt.), 1/15th LD_{50} (2.47 mg/kg body wt) and 1/10th LD_{50} (3.70 mg/kg body wt.). The animals were sacrificed on day 18. All foetuses were examined and a portion of the liver of two from each set were dissected out, minced finely and fixed in 1:3 acetic ethanol (three changes). Slides were