Marked Delay in Excystment of *Schizopyrenus russelli* Cysts by *p*-Chloromercuric Benzoate and its Reversal with Reduced Glutathione

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SUMMARY

p-Chloromercuric benzoate (*p*-CMB) caused marked delay in excystment of *Schizopyrenus russelli* cysts when incorporated in the aqueous extract of *Escherichia coli*, an excystment agent. Cysts treated with *p*-CMB first and then with reduced glutathione (GSH), excysted readily in the presence of bacterial extract, after the removal of *p*-CMB and GSH.

INTRODUCTION

Actively proliferating bacteria of an *Aerobacter* sp. and *Escherichia coli* and their aqueous extracts cause excystment of cysts of *Schizopyrenus russelli*; certain pure amino acids, within a suitable pH range, also cause excystment (Singh, Mathew & Anand, 1958). Further, the excystment-inducing property of glutamic acid and of aqueous extract of *E. coli* was completely inhibited by emetine hydrochloride. This inhibition was completely annulled after the removal of emetine (Imam, Dutta & Agarwala, 1968).

The present communication deals with the delay in excystment of *Schizopyrenus russelli* cysts caused by *p*-chloromercuric benzoate and its reversal with reduced glutathione.

METHODS

Preparation of viable sterile cysts of Schizopyrenus russelli. The cysts from a 'pure line' culture of the amoeba, growing with *Escherichia coli*, were used. Cysts free from living and dead bacteria were obtained essentially according to the method of Singh, Saxena & Iyer (1965). Non-nutrient agar, containing agar 3.5 and NaCl 0.5 (% w/v), pH 6.8 to 7.0, in 11 Roux-type culture flasks was seeded with cysts (1×10^6) and a thick suspension of *E. coli* in 0.5 % (w/v) NaCl. After 12 to 15 days, at 25°, the cysts were harvested and washed with distilled water several times by centrifugation to get rid of most of the bacteria. They were then treated with N-HCl for 24 hr at room temperature to kill bacteria, active amoebae and partially formed cysts. The acid was removed by repeated washing with water. The thick suspension of cysts thus obtained was treated for a few minutes with a sterile solution of trypsin (2 %, w/v) to lyse dead bacteria completely. The cysts were washed quickly several times with distilled water to get rid of small particles and water-soluble products released from the dead bacteria. After testing the bacterial sterility of the cysts, they were diluted to 10×10^6 /ml., counted with a haemocytometer.

Bacterial extract. Eighteen hours growth of *Escherichia coli* on nutrient agar (NaCl 0.5 %, peptone 1 %, Marmite 0.1 % and Lab-Lemco 0.3 %, pH 7.2) was harvested with physiological saline and washed twice by centrifugation. The pellet was weighed and, for every g. wet bacteria, 5 ml. of distilled water were added. The suspension was autoclaved at 121° for 15 min., thereafter it was centrifuged aseptically. The clear straw-coloured supernatant fluid (pH 6.5) was stored in a refrigerator and used as the excystment agent.

Excystment procedure. To 0.1 ml. of cyst suspension in a sterile culture tube (10 × 1.2 cm.) were added 0.7 ml. of bacterial extract, followed by 0.1 ml. *p*-CMB solution + 0.1 ml. water in experimental and 0.2 ml. distilled water in control experiment. The tubes were incubated in inclined position at 25°. Samples were taken at regular intervals and counts of the different stages of excystment were made with a haemocytometer. *Schizopyrenus russelli* forms a double-walled cyst and excystment takes place in two stages. First, the inner wall disappears and a small amoeba moves freely within the outer wall. In a successful case, the outer wall gives way and the amoeba emerges.

In pretreatment experiments the same quantity of cysts were incubated with 0.1 ml. of *p*-CMB solution and/or 0.1 ml. reduced glutathione (final volume 1 ml.) for 1 hr, after which cysts were washed 3 times with distilled H₂O and then studied for excystment in bacterial extract, as given earlier. Proper controls were run in all the experiments.

RESULTS

The data presented in Table 1 showed that *p*-CMB, at 10⁻³M and 5 × 10⁻³M, completely prevented the emergence of trophozoites from the cysts up to 6 hr. At 10⁻⁴M the excystment in 4 and 6 hr was decreased. At 6 hr in the tubes with 10⁻⁴M *p*-CMB,

Table 1. *Effect of p-chloromercuric benzoate (M) on the excystment of Schizopyrenus russelli cysts*

Stage:	2 hr			4 hr			6 hr		
	A*	B*	C*	A	B	C	A	B	C
Concn. (M)	Count (%)								
Control	43	19	38	15	22	63	10	25	65
5 × 10 ⁻³	95	5	0	91	9	0	86	14	0
10 ⁻³	90	10	0	89	11	0	82	18	0
10 ⁻⁴	36	54	10	9	82	9	14	60	26

* A, Intact cysts; B, intermediary stage (trophozoite moving within the outer cyst wall); C, trophozoites.

60 % of the cysts were in the intermediary stage of excystment, but at higher concentrations of *p*-CMB there was a considerable delay in the appearance of intermediary stages and only 14 to 18 % cysts were in that stage. Table 2 shows that 81 % of cysts pretreated with *p*-CMB (10⁻³M) for 1 hr were in the intermediary stage of excystment after 4 hr which increased to 93 % in 8 hr; but the trophozoites failed to hatch. *p*-CMB at 10⁻⁴M, which decreased excystment when present throughout the experiment, had no permanent effect since excystment paralleled the control when *p*-CMB was removed.

Although *p*-CMB at 10^{-3} M and 5×10^{-3} M completely prevented the emergence of trophozoites from the cysts up to 6 to 8 hr, nearly all the cysts excysted in 18 to 24 hr, as in the control. Hence all the cysts were viable. The data presented in Table 3 show that, when cysts were treated with *p*-CMB and reduced glutathione (GSH) together, there was 90 % excystment in 4 hr, as compared with none when incubated with *p*-CMB alone. There was 76 % excystment in 4 hr when the cysts were first treated with *p*-CMB for 1 hr and then with GSH for 1 hr, as against 81 % in the control.

Table 2. *Effect of pretreatment of cysts of Schizopyrenus russelli with p-chloromercuric benzoate on their subsequent excystment*

The cysts were treated with *p*-chloromercuric benzoate for 1 hr, washed thrice with distilled water and then left for excystment.

Stage:	2 hr			4 hr			6 hr			8 hr		
	A*	B*	C*	A	B	C	A	B	C	A	B	C
Concn. (M)	Count (%)											
Control	60	8	32	13	21	66	2	8	90	1	4	95
10^{-3}	65	35	0	19	81	0	16	84	0	7	93	0
10^{-4}	72	4	24	34	7	59	6	3	91	4	0	96

* A, B, C as in Table 1.

Table 3. *Effect of reduced glutathione in reversing the delay in excystment of Schizopyrenus russelli cysts caused by p-CMB*

The cysts after each treatment were washed thrice with distilled water and then put for excystment.

Stage:	2 hr			4 hr		
	A*	B*	C*	A	B	C
Concn. (M)	Count (%)					
Control	34	4	62	14	5	81
Pretreated with <i>p</i> -CMB for 1 hr (10^{-3} M)	95	5	0	47	53	0
Pretreated with <i>p</i> -CMB (10^{-3} M) + GSH (2×10^{-3} M) for 1 hr	13	0	87	10	0	90
Pretreated with <i>p</i> -CMB (10^{-3} M) for 1 hr and then with GSH (2×10^{-3} M) for 1 hr.	45	14	41	24	0	76

* A, B, C as in Table 1.

DISCUSSION

p-CMB is a specific inactivator of $-SH$ groups (Webb, 1966). The present studies definitely indicate involvement of such groups in the process of excystment of cysts of *Schizopyrenus russelli*. *p*-CMB presumably interferes with enzyme function during excystment and that interference is completely annulled by reduced glutathione. The initiation of excystment does not appear to be as sensitive to the action of *p*-CMB as the final emergence of the trophozoites.

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