Chloramphenicol stimulates acid phosphatase activity in germinating cotton (*Gossypium hirsutum*) embryos

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Low dosages of chloramphenicol (25–50 µg/ml) brought about a 2–4-fold stimulation of acid phosphatase activity in 48 h-germinated cotton (*Gossypium hirsutum*) embryos. However, at high concentrations of chloramphenicol (100–1000 µg/ml), there was a progressive decline in enzyme activity. The stimulatory effect of the drug on acid phosphatase activity was relatively specific, since no significant stimulation of activities of proteinase, deoxyribonuclease, ribonuclease, *o*-diphenolase and peroxidase was observed in germinating cotton embryos. Chloramphenicol, however, did promote the activities of isocitric lyase and alkaline phosphatase. Sephadex G-200 chromatography of the enzyme fraction revealed high (230 000)- and low (106 000)-molecular-weight multiple forms of acid phosphatase in the chloramphenicol-treated embryos, in contrast with a single molecular form (mol.wt. 106 000) in the untreated embryos. Thus the treatment of cotton embryos with chloramphenicol induced both a qualitative and a quantitative change in the acid phosphatase activity. Chloramphenicol-stimulated acid phosphatase activity was strongly inhibited when P_i was included in the germination medium. However, the control embryos showed less pronounced inhibition of enzyme activity in presence of P_i ions.

Chloramphenicol is a potent inhibitor of protein synthesis in prokaryotes (Sypherd *et al.*, 1962) and in eukaryotic plastids (Gale & Folkes, 1953). However, application of this antibiotic to plant and bacterial cells is known to promote the activity of a few enzymes. A 4-fold stimulation of nitrate reductase activity by chloramphenicol was reported in *Staphylococcus aureus*. The drug, however, brought about an inhibition (about 88%) of [^{14}C]arginine incorporation into the protein fraction (Ramsey, 1966). Chloramphenicol treatment also stimulated nitrate reductase activity in corn (*Zea mays*) (Schrader *et al.*, 1967) and rice (*Oryza sativa*) seedlings (Shen, 1972). Shen (1972) speculated that the nitro group of the antibiotic could be responsible for the induction of enzyme activity. Knypl (1973) observed enhancement of nitrate reductase activity in chloramphenicol-treated excised cucumber (*Cucumis sativus*) cotyledons. The stimulatory effect of the drug was nullified by cycloheximide.

Stereospecific effects of chloramphenicol have been observed both in bacteria and in higher plants. Out of the two stereoisomers of chloramphenicol (*L*-threo and *D*-threo), only *D*-threo-chloramphenicol inhibited protein synthesis of chloro-

plast ribosomes in tobacco (*Nicotiana tabacum*) leaves (Ellis, 1969). The drug also inhibited the activity of ribulose biphosphate carboxylase and phosphoribulokinase in green leaves of French bean (*Phaseolus vulgaris*) (Ireland & Bradbeer, 1971). However, the activity of transketolase was stimulated by *D*-threo-chloramphenicol in primary leaves of *Phaseolus vulgaris* (Ireland & Bradbeer, 1971).

In the present study we observed a 2–4-fold stimulation of acid phosphatase activity by chloramphenicol in germinating cotton embryos. The drug-induced enzyme activity was strongly inhibited by the addition *in vivo* of phosphate ions. In addition, the chloramphenicol-treated embryos were shown to contain an additional molecular form of acid phosphatase.

Experimental

Reagents

Chloramphenicol was a product of Parke–Davis, Bombay, India. Yeast RNA and isocitric acid were purchased from Sigma, St. Louis, MO, U.S.A. Sephadex G-25 and Sephadex G-200 were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden.

o-Dianisidine hydrochloride and *p*-nitrophenyl phosphate were purchased from Biochemical Unit, V.P. Chest Institute, Delhi, India. The marker proteins (ribonuclease A, chymotrypsinogen A, ovalbumin, bovine serum albumin and aldolase) for molecular-weight determination were procured from Pharmacia. All other buffers, salts and chemical reagents were of analytical grade.

Cotton (*Gossypium hirsutum* var. F. 414) seeds were procured from the Punjab Agricultural University, Ludhiana, India, and the Indian Agricultural Research Institute, Sirsa, India.

Germination of embryos

The seeds were treated with 3 M-HCl (10 min) to inactivate the fungal spores entangled in the 'fuzz'. The acid was removed by repeated washing with water and the seeds were air-dried before storage. Before germination, the seed coat was removed manually after soaking the seeds in distilled water for 30 min at 25°C. The excised embryos were surface-sterilized with aq. 0.02% HgCl₂ for 4 min and rinsed several times with sterile distilled water. The embryos were germinated in dark at 25°C under aseptic conditions on autoclaved agar (0.4%) medium in the absence and presence of chloramphenicol (25–1000 µg/ml). P₁ (5 mm or 10 mm) was added to control and chloramphenicol-treated embryos during germination for testing its effect on acid phosphatase activity.

Enzyme preparation

The germinated cotton embryos were homogenized in chilled (4°C) sodium phosphate buffer (0.1 M, pH 6.6; 1 g of tissue/20 ml of buffer) containing β-mercaptoethanol (0.14 mM) and Celite (2 g). The homogenate was filtered through four layers of muslin and centrifuged at 27000 *g* for 15 min in the cold (4°C). The supernatant was filtered through a layer of absorbent cotton to remove the fatty layer. The dialysed crude extract was employed for the assay of acid phosphatase activity.

Crude extract prepared from control and chloramphenicol-treated embryos (48 h) was enriched by precipitation with solid (NH₄)₂SO₄ (0–65% satn.) and desalted on a Sephadex G-25 column (18 cm × 1.8 cm). This fraction was also tested for acid phosphatase activity.

Assay of acid phosphatase activity

A slightly modified procedure (Johnson *et al.*, 1973) was adopted for the assay of acid phosphatase activity. The incubation mixture comprised acetate buffer (225 µmol, pH 5.0), *p*-nitrophenyl phosphate (5.0 µmol) and enzyme fraction (0.4–2.0 mg of protein), in a final volume of 4 ml. After incubating the assay mixture for 30 min at 37°C, a small portion (0.5 ml) of the reaction mixture was

added to dilute alkali solution (0.1 M-NaOH, 5.5 ml) for developing the colour of the *p*-nitrophenol product. The A₄₀₅ of the resultant solution was measured. A unit of acid phosphatase activity is defined as the amount of enzyme which liberates 1 nmol of *p*-nitrophenol in 30 min in an incubation mixture of 4.0 ml.

Assay of other enzymes

The procedures of Beevers (1968) and Rosen (1957) were used for the assay of proteinase activity. Ribonuclease and deoxyribonuclease activities were measured as described by Scrubb *et al.* (1972). Isocitric lyase activity was assayed by the procedure of Smith *et al.* (1974) and *o*-diphenolase activity was determined as described by Taneja & Sachar (1974). Peroxidase activity was measured by using a modification of the procedure of Shannon *et al.* (1966). The method of Dorn & Rivera (1966) was used for measuring the activity of alkaline phosphatase.

Molecular-sieve chromatography on Sephadex G-200

The 0–65%-satd.-(NH₄)₂SO₄ precipitate prepared from control and chloramphenicol-treated embryos (48 h) was desalted on a Sephadex G-25 column (18 cm × 1.8 cm). A portion (2.5 ml, containing 7.5 mg of protein) of this fraction was loaded on a Sephadex G-200 column (72 cm × 1.8 cm) pre-equilibrated with degassed phosphate buffer (50 mM, pH 6.6). The void volume (*V*₀ = 54.0 ml) of the gel column was determined experimentally from the elution profile of Blue Dextran. The specific activity of acid phosphatase was plotted against the fraction number. The molecular weights of the multiple forms of acid phosphatase were estimated from their distribution coefficients (*K*_{av}). Marker proteins (ribonuclease A, chymotrypsinogen A, ovalbumin, bovine serum albumin and aldolase) were used for the calibration of the Sephadex column.

Protein determination

Protein was determined as described by Lowry *et al.* (1951).

Results

Stimulatory effect of chloramphenicol on acid phosphatase activity

Dialysed crude extracts prepared from 30 min-imbibed cotton embryos showed negligible acid phosphatase activity (395 enzyme units). A 10-fold increase in acid phosphatase activity (4105 enzyme units) was witnessed in embryos germinated for 48 h. The enzyme activity showed a further rise (36-fold) in 72 h-old seedlings (14545 enzyme units; see Fig.

Fig. 1. Time-course studies showing the stimulatory effect of chloramphenicol on acid phosphatase activity in germinating cotton embryos

Embryos were germinated in the dark at $25 \pm 2^\circ\text{C}$ on 0.4% agar medium for different intervals of time (24, 48 and 72 h) in the absence (○) or presence (●) of chloramphenicol ($50\mu\text{g/ml}$). Acid phosphatase activity was assayed in dialysed crude extracts.

Fig. 2. Effect of different concentrations of chloramphenicol on acid phosphatase activity in germinating cotton embryos

Cotton embryos were germinated in the dark at $25 \pm 2^\circ\text{C}$ for 48 h on 0.4% agar medium containing different concentrations for chloramphenicol (25–1000 μg/ml). Acid phosphatase activity was measured in dialysed crude extracts of control and chloramphenicol-treated embryos.

1). Embryos germinated in the presence of chloramphenicol ($50\mu\text{g/ml}$) brought about a 2.7-fold stimulation of acid phosphatase activity over the untreated controls in 72 h-germinated embryos (Fig. 1). The stimulation of acid phosphatase activity by chloramphenicol was quite comparable whether the enzyme units were expressed on a per-embryo or on a per-mg-of-protein basis. The stimulation of acid phosphatase activity (2.72-fold) was also witnessed in the 0–65%-satd.-(NH_4) $_2\text{SO}_4$ -precipitated fraction prepared from chloramphenicol-treated embryos (results not shown). This excluded the possibility of enzyme activation by chloramphenicol. Fig. 2 depicts the activity profile of acid phosphatase as a function of chloramphenicol concentration (25–1000 μg/ml). It was evident that low dosages of the drug (25–50 μg/ml) stimulated acid phosphatase activity. However, at high concentrations of chloramphenicol (100–1000 μg/ml), there was a progressive decline in the stimulatory response of the drug.

In order to ascertain the specificity of chloramphenicol towards acid phosphatase activity, we tested the effect of the drug on various enzymes. We observed no appreciable increase in the activities of

proteinase, deoxyribonuclease and ribonuclease in chloramphenicol-treated embryos. A slight stimulatory response was witnessed in *o*-diphenolase and peroxidase. However, a significant stimulation of alkaline phosphatase, acid phosphatase and isocitric lyase was observed in chloramphenicol ($50\mu\text{g/ml}$)-treated embryos over that of the control (Table 1). Thus the effect of the drug does not seem to be absolutely specific for phosphatases in germinating cotton embryos.

End-product repression of acid phosphatase activity

Germination of cotton embryos in phosphate buffer (pH 6.6, 5–10 mM) medium showed a differential inhibitory response on acid phosphatase activity in control and chloramphenicol-treated embryos. Whereas a strong repression (91% inhibition of acid phosphatase activity) was observed in chloramphenicol-treated embryos at a concentration of 5 mM- P_i , there was virtually no inhibition of enzyme activity in control embryos at this concentration. However, a significant repression of acid phosphatase activity (57% inhibition) was observed in control embryos at a higher concentration of P_i (10 mM; see Table 2).

Table 1. *Effect of chloramphenicol on proteinase, deoxyribonuclease, ribonuclease, o-diphenolase, peroxidase, isocitric lyase, alkaline phosphatase and acid phosphatase activities in germinating cotton embryos*
Cotton embryos were germinated in the dark at $25 \pm 2^\circ\text{C}$ for 48 h on a 0.4% agar medium in the absence and presence of chloramphenicol ($50 \mu\text{g/ml}$). The enzyme activities were assayed in dialysed crude extracts.

Enzyme	Enzyme activity (units/mg of protein)		Stimulation over control (%)
	Control	Chloramphenicol ($50 \mu\text{g/ml}$)	
Proteinase	266.0	269.0	1
Deoxyribonuclease	1295.0	1417.50	9
Ribonuclease	2200.0	2450.0	11
<i>o</i> -Diphenolase	0.143	0.182	27
Peroxidase	70.0	95.7	36
Isocitric lyase	25.78	56.23	118
Alkaline phosphatase	755.0	1785.0	136
Acid phosphatase	2946.08	11776.46	299

Table 2. *Differential inhibitory effect of P_i on acid phosphatase activity in control and chloramphenicol (CAP)-treated embryos*

Cotton embryos were germinated in the dark at $25 \pm 2^\circ\text{C}$ for 48 h on 0.4% agar medium in the absence (a) or presence (b) of chloramphenicol ($50 \mu\text{g/ml}$). P_i (sodium phosphate buffer, pH 6.6, at 5 mM or 10 mM) was added to the medium at the inception of germination. Acid phosphatase activity was measured in the dialysed crude extracts.

Addition(s)	Acid phosphatase activity	
	(units/mg of protein)	(% of control)
(a) Control	1057	100.0
P_i 5 mM	1042	98.5
P_i 10 mM	453	42.8
(b) CAP		
CAP ($50 \mu\text{g/ml}$)	6100	100.0
CAP ($50 \mu\text{g/ml}$) + P_i (5 mM)	547	8.9
CAP ($50 \mu\text{g/ml}$) + P_i (10 mM)	630	10.3

Physical separation of multiple forms of acid phosphatase

The multimeric nature of acid phosphatase was determined by fractionating the enzyme preparations by molecular-sieve chromatography. Gel filtration of the 0–65%-satd.-(NH_4)₂SO₄-precipitated fraction on a column (72 cm $\times$ 1.8 cm) of Sephadex G-200 revealed two forms (peaks I and II) of acid phosphatase in chloramphenicol-treated embryos,

Fig. 3. *Separation of multiple forms of acid phosphatase in control and chloramphenicol-treated embryos*

Cotton embryos were germinated in the dark for 48 h at $25 \pm 2^\circ\text{C}$ on 0.4% agar medium. 0–65%-satd.-(NH_4)₂SO₄-precipitated fractions were prepared from control (O) and chloramphenicol ($50 \mu\text{g/ml}$)-treated (●) embryos and were desalted on a column (18 cm $\times$ 1.8 cm) of Sephadex G-25. A portion (2.5 ml, containing 7.5 mg of protein) of these fractions was fractionated on a column (72 cm $\times$ 1.8 cm) of Sephadex G-200. The small unlabelled arrow to the left of the Figure represents the void volume ($V_0 = 54 \text{ ml}$) of the column. The enzyme activity and concentration of the protein were determined in each fraction (4 ml).

whereas a single molecular form (peak II) of the enzyme was observed in control embryos (Fig. 3). The activity peak I represented a high-molecular-weight (230 000) multiple form, whereas the activity peak II represented a low-molecular-weight (106 000) form. The elution profile of peak II in controls coincided with that of the activity peak II in chloramphenicol-treated embryos (Fig. 3). Thus the treatment of embryos with chloramphenicol not only increased the specific activity of acid phosphatase, but also induced an additional multiple form.

Discussion

The induction of several hydrolytic enzymes has been studied during the early germination of cotton embryos. Dure and his associates (Dure, 1975; Dilworth & Dure, 1978) have reported the presence

of conserved messenger-RNA species in cotton embryos that can support the synthesis of isocitric lyase, carboxypeptidase, arginase, urease and glutamate dehydrogenase during early stages of embryo germination. The evidence in support of this hypothesis was based on inhibitor studies with actinomycin D. Smith *et al.* (1974) consider that there is not enough evidence to propose the presence of conserved message for isocitric lyase in cotton embryos, since administration of actinomycin D and cordycepin to the germinating cotton embryos resulted in a strong inhibition of this enzyme.

An unusual feature observed by us in the present study was the stimulation (2–4-fold) of acid phosphatase activity when the cotton embryos were germinated in the presence of chloramphenicol (25–50 µg/ml). The stimulatory effect was, however, not witnessed at higher concentrations (100–1000 µg/ml) of the drug. Although chloramphenicol stimulated the activities of nitrate reductase and alcohol dehydrogenase in other plant tissues (see Ramsey, 1966; Schrader *et al.*, 1967; Shen, 1972; Knypl, 1973; Plas *et al.*, 1981; Vartapetyan *et al.*, 1975), virtually nothing is so far known about the possible mode of action of this drug. Our study has now revealed some new facts about the effect of chloramphenicol on acid phosphatase in germinating cotton embryos. These are as follows. (i) Chloramphenicol is not serving as an activator, since the dialysed crude extract and the $(\text{NH}_4)_2\text{SO}_4$ -precipitated fraction showed enhancement of enzyme activity over the untreated controls. (ii) The stimulatory effect of the drug was not only restricted to acid phosphatase, since an enhancement of enzyme activity was also observed for isocitric lyase and alkaline phosphatase. Other enzymes, such as proteinase, deoxyribonuclease and ribonuclease, showed no stimulatory response to chloramphenicol. However, a slight increase in the activities of *o*-diphenolase and peroxidase was observed in chloramphenicol-treated embryos. (iii) The drug-induced acid phosphatase activity was strongly repressed by addition of P_i to the germinating embryos. (iv) Molecular sieving of enzyme preparation on Sephadex G-200 showed an additional high-molecular-weight (230 000) form in chloramphenicol-treated cotton embryos. This form was completely absent from control embryos. The additional form in chloramphenicol-treated em-

bryos could have arisen by the molecular aggregation of smaller subunit(s) of acid phosphatase. In view of the above observations, we propose that the drug is in some manner responsible for the induction of acid phosphatase activity. However, the precise site of action of chloramphenicol in enzyme regulation requires further probing.

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