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THE EFFECTS OF PROTEIN SYNTHESIS INHIBITORS ON OXIDATIVE PHOSPHORYLATION BY PLANT MITOCHONDRIA

S. B. WILSON and A. L. MOORE

Department of Biochemistry, University of Aberdeen, Marischal College, Aberdeen, AB9 1AS (Great Britain)

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SUMMARY

Inhibitors can be successfully used if they are specific for only one process. Published data suggest that some inhibitors of protein synthesis may also inhibit respiration or oxidative phosphorylation. The effect of a range of protein synthesis inhibitors on respiration and phosphorylation has been studied, using tightly coupled mitochondria from several plant species including turnips (*Brassica napus*).

Puromycin, actinomycin D, lincomycin, mitomycin C and D-serine did not uncouple or inhibit respiration. Cycloheximide caused a partial inhibition (maximum 22% at 3 mM) of malate but not succinate-driven respiration. Chloramphenicol was a potent inhibitor of electron transport, but not of phosphorylation. The activity of the isomers of chloramphenicol varied in the order L-*threo* > D-*threo* > L-*erythro* > D-*erythro*. From evidence presented it is concluded that chloramphenicol has three sites of action, the flavoprotein level being most sensitive, the second site of variable sensitivity lies between cytochromes *b* and *c* and the third site at the cytochrome *a* level is only slightly affected by the inhibitor.

INTRODUCTION

The conclusion that subcellular organelles are autonomous is based on the widespread use of inhibitors of protein synthesis and depends on the specificity of the inhibitors employed. This specificity of action, assumed in many studies, is particularly important when isolated organelles, crude homogenates or *in vivo* systems are the subject of study. High concentrations of inhibitor are often added to ensure that adequate levels of inhibitor are present at the site of action.

Several observations suggest that not all the commonly used inhibitors of protein synthesis are specific for the process, particularly when the inhibitors are applied to plant protein synthesis systems. Thus, D-serine, a competitive inhibitor for L-serine, has been observed to inhibit ion uptake in plant tissues. Cycloheximide[2,3] stimulates oxygen uptake and inhibits ion uptake by a number of plants, suggesting that it may uncouple oxidative phosphorylation. A number of investigations have shown that chloramphenicol is not always specific in its

Abbreviations: 1799, bis-hexafluoroacetonyl acetone; TMPD, *N,N,N',N'*-tetramethylene-*p*-phenylenediamine.

action. Inhibition of ATP formation in rat reticulocytes[4] and of NAD-linked oxidations in beef heart mitochondria[5,6] result from the use of chloramphenicol. Salt uptake by various intact plant tissues[7,8] and ion uptake and oxidative phosphorylation by isolated maize mitochondria[9–11] are also inhibited by chloramphenicol. Electron transport in *Microbacterium phleii* is, however, not affected[12] by concentrations of chloramphenicol which inhibit protein synthesis. The concentrations used were however lower than those employed in experiments where inhibition of electron transport and phosphorylation occurred.

Observations of a lack of specificity with chloramphenicol and cycloheximide have not involved the use of tightly coupled mitochondria. This paper describes a study of the effects of a range of protein synthesis inhibitors on respiration and phosphorylation by tightly coupled plant mitochondria. Inhibitors studied were, chloramphenicol, cycloheximide, D-serine, lincomycin, puromycin, mitocmyin C and actinomycin D[8,13,14].

MATERIALS AND METHODS

Antibiotics were obtained from commercial sources: puromycin from Nutritional Biochemicals Ltd, Cleveland, Ohio, U.S.A., lincomycin from Upjohn and Co., Kalamazoo, Michigan, U.S.A., actinomycin D from Makor Chemicals Ltd, Jerusalem, Israel, mitomycin C in 0.3 M NaCl, cycloheximide and D-*threo*-chloramphenicol from Sigma Chemical Co., St. Louis, Mich., U.S.A., D-serine and L-serine from Koch Light Laboratories, Colnbrook, Bucks., U.K., D-*erythro*-, L-*erythro*- and L-*threo*-chloramphenicol were the gift of Dr R. J. Ellis, University of Warwick, U.K. Bis-hexafluoroacetonyl acetone (1799), a potent uncoupler of oxidative phosphorylation, was the gift of Dr P. Heytler, Du Pont de Nemours Ltd, Wilmington, Dela., U.S.A. Inhibitors were dissolved in water, ethanol or dimethyl sulphoxide and added to the reaction cuvette in small aliquots when required. Control experiments using organic solvents showed they had no significant effects at the levels used in this study. All the other reagents were reagent grade material obtained from commercial sources.

Mitochondria were prepared from turnip roots (*Brassica napus*), cauliflower buds (*Brassica oleracea* c.v. *botrytis*) and potatoes (*Solanum tuberosum*), purchased locally, or from etiolated french bean hypocotyls (*Phaseolus vulgaris* c.v. Canadian Wonder) and Maize (*Zea mays*) coleoptiles grown in the laboratory.

After preliminary chopping, tissues were homogenised with a "Polytron" homogeniser and the mitochondria isolated as described by Ikuma and Bonner[15] except that the preliminary low-speed centrifugation was replaced by sieving through a nylon cloth of approximately 50-μm pore size. Mitochondria were only used for experiments if they showed good respiratory control ratios and ADP:O values (respiratory control > 2.5 and ADP:O 1.6 with succinate and respiratory control > 3.5 and ADP:O 2.3–2.5 with malate as substrate), values normally observed with intact plant mitochondria[15]. State 3 (ref. 16) oxygen uptake rates were 140–220 nmoles O_2/mg protein per min with succinate or malate as substrate.

Oxygen consumption was measured with a Clark type oxygen electrode in a magnetic stirred "Plexiglas" cuvette of 3 ml capacity containing 0.5–1 mg/ml mitochondrial protein. The oxygen uptake was calculated on the basis of 240 μM

oxygen in air-saturated medium. Measurements of the effects of inhibitor were made when any new rate of oxygen uptake had been established for 1 min, experiments lasted no longer than 5–7 min.

The reaction mixture contained 0.3 M mannitol. 10 mM KCl, 5 mM $MgCl_2$, 10 mM potassium phosphate buffer, pH 7.2, with 16 mM malate, 8 mM succinate, 1.6 mM NADH or 10 mM ascorbate, 1 mM *N,N,N',N'*-tetramethylene-*p*-phenylenediamine (TMPD) mixture as alternative substrates. 0.16–0.5 mM ADP was added to initiate State 3 (ref. 16) when required, State 4 (ref. 16) was induced when the ADP was depleted or by 0.16 mM ATP.

Phosphate uptake measurements were made using a medium containing 2.5 mM potassium phosphate with 3 mM glucose and 0.15 mg/ml hexokinase as an ATP trap. Phosphate concentrations of subsamples from the reaction cuvette were measured colorimetrically[17] after stopping the reaction and precipitating the protein with 10% trichloroacetic acid.

Difference spectra were obtained in a specially constructed split-beam spectrophotometer[18]. Steady state reduction levels of the redox carriers; flavoprotein, cytochrome *b*, cytochrome *c* and cytochrome *a* were measured using a double-beam spectrophotometer[19] with 470–520, 560–540, 550–540 and 605–620 nm as wavelength pairs, respectively. The carriers were assumed to be totally oxidised after depletion of the mitochondria for 5 min with 0.16 mM ADP and fully reduced after the addition of a small amount of dithionite.

RESULTS AND DISCUSSION

Lincomycin, puromycin, mitomycin C and actinomycin D were without significant effect on turnip mitochondria at the concentrations normally used in experiments on protein synthesis. Any small effects at much higher concentrations being comparable to those obtained by using the solvent alone. The effects of D-serine were also very small and matched by L-serine. 2 μM cycloheximide resulted in a 10% inhibition of malate but not succinate oxidation, in State 3, with a maximum inhibition of 22% even when the concentration was raised to 3 mM. This result suggests that cycloheximide may act at the flavoprotein level of the electron transport system. None of the inhibitors significantly stimulated State 4 oxygen uptake indicating that they lack uncoupler-like activity. It may be concluded that, with the exception of cycloheximide, inhibition of respiration and energy conservation is not likely to accompany the use of these inhibitors in short-term protein-synthesis experiments.

In contrast to the other inhibitors used, D-*threo*-chloramphenicol had a marked affect on the oxidation of malate in State 3 with turnip mitochondria. The half maximum inhibitory concentration was 2 mM (Fig. 1B). Inhibition of succinate and NADH oxidations and of State 4 (Fig. 2), was less marked. Comparative lack of inhibition of NADH oxidation was not due to competition of NAD with the inhibitor for a site of action since lower NAD concentrations kept reduced with alcohol dehydrogenase were equally insensitive to the inhibitor. This result agrees with the hypothesis that exogenous NADH oxidation uses a different pathway to that of endogenous NADH oxidation. 10 min pretreatment of mitochondria in State 4 with 1799 resulted in an inhibitor sensitivity similar to

that for State 3. Addition of oligomycin to mitochondria in State 3 converted the inhibition to the State 4 type. Ascorbate TMPD oxidation, which uses only the cytochrome *c* to oxygen segment of the respiratory chain, was not inhibited by D-*threo*-chloramphenicol. The differing sensitivity of the various substrate oxidations to the drug is not explicable on the relative rates of oxidation, since malate and succinate are oxidised at approximately equal rates. Fig. 2 shows that the inhibition of State 4 malate oxidation did not occur until the inhibitor concentration

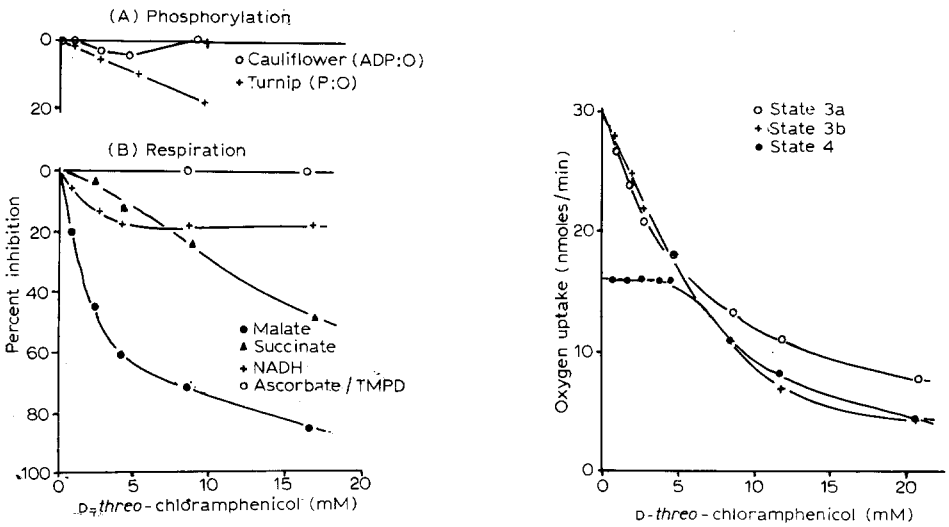

Fig. 1. Inhibition of mitochondrial respiration and phosphorylation by D-*threo*-chloramphenicol. Results expressed as percentage of control value. (A) Inhibition of cauliflower ADP:O and turnip P:O values with malate as substrate. (B) Inhibition of State 3 respiration of turnip mitochondria. Experimental conditions as described in the text.

Fig. 2. Inhibition by D-*threo*-chloramphenicol of turnip mitochondrial respiration with malate as substrate. The inhibitor was added approximately 1 min after inducing a State 3 cycle with 0.16 mM ADP. The inhibitor measured prior to the exhaustion of the ADP is designated State 3a. The State 4 inhibition was measured after the ADP was depleted and the State 3b inhibition after a further addition of 0.16 mM ADP. Results expressed with reference to the control value.

was sufficient to eliminate ADP stimulation. Fig. 2 also indicates a delay in obtaining complete inhibition of the State 3 oxidation of malate at higher concentrations of inhibitor. While inhibition seemed complete within 15 s of adding D-*threo*-chloramphenicol a further decline in the oxidation rate occurred on the exhaustion of a limiting amount of ADP. An additional slight decline sometimes occurred after a further ADP addition.

While mitochondria from turnips were usually much less sensitive to D-*threo*-chloramphenicol with succinate as substrate, it was noted that succinate oxidation was more sensitive during the spring when the turnips start to produce a flowering spike. It was subsequently found that other sources of plant mitochondria had differing sensitivities to D-*threo*-chloramphenicol with succinate as substrate (Fig. 3). These results suggest that there may be a site of inhibition with a variable sensitivity between succinate and cytochrome *c*.

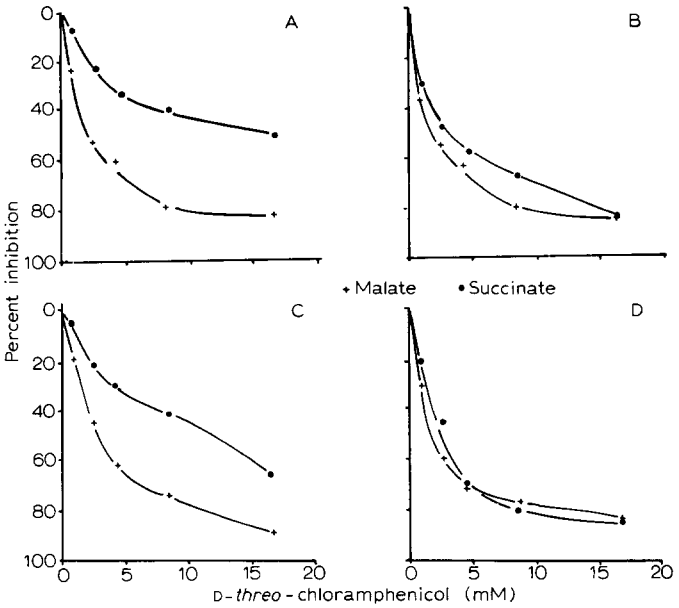

Fig. 3. Inhibition of malate and succinate State 3 respiration by D-*threo*-chloramphenicol. Mitochondria from: (A) Maize. (B) French bean. (C) Cauliflower. (D) Potato. Conditions as described in the text.

Contrary to the results reported for other plant systems[7–11], no evidence for a marked inhibition of phosphorylation was obtained in this study. Fig. 1A shows D-*threo*-chloramphenicol caused only a 20% reduction in P:O ratio with turnip mitochondria and no reduction of ADP:O ratio with cauliflower bud mitochondria. No marked stimulation of State 4 respiration was recorded. This contrast with published data probably results from the short times required to perform oxygen electrode experiments compared with the times involved in using other methods. The results reported in this paper are direct measurements of phosphorylation, unlike the measurements of salt uptake and swelling reported elsewhere.

A comparison of the effect of the isomers of chloramphenicol on malate oxidation in State 3 showed (Fig. 4) that all caused some inhibition, the effect being in the order L-*threo* > D-*threo* > L-*erythro* > D-*erythro*. Identical results were obtained using succinate or NADH as substrates. Of these isomers only D-*threo*-chloramphenicol is an effective inhibitor of protein synthesis in plant organelles[20]. These results support the suggestion that L-*threo*-chloramphenicol should be used in parallel control experiments if inhibition of protein synthesis *per se* is to be differentiated from any other inhibition.

Oxidised–reduced difference spectra of turnip mitochondria obtained at 77 °K support the conclusion that there are two sites of action of chloramphenicol. Fig. 5 shows that the spectrum obtained 5 s after adding a small amount of D-*threo*-chloramphenicol is dominated by a large flavoprotein trough centred around 470 nm with a triple peak at 552–557 and 561 nm corresponding to the three

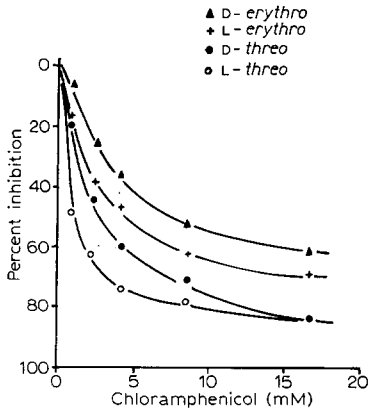

Fig. 4. Inhibition by chloramphenicol isomers of State 3 turnip mitochondrial respiration with malate as substrate. Experimental conditions as described in the text.

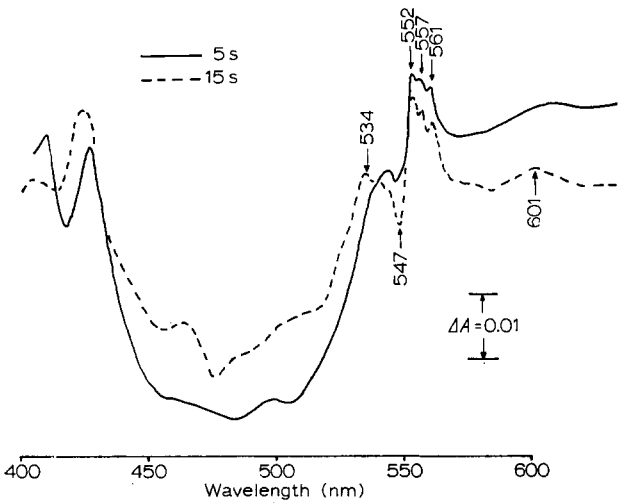

Fig. 5. Difference spectra recorded at 77 °K. Malate State 3–malate State 3+330 μM D-*threo*-chloramphenicol for 5 or 15 s before freezing. Approximately 2 mg/ml turnip mitochondria. Light path, 0.2 cm. Media as described in the text.

cytochromes *b* normally found in plant mitochondria. Exposure to the drug for 15 s or to higher concentrations increased the size of the cytochrome *b* peaks and induced peaks at 534 and 601 nm. These represent a further reduction of the *b* type cytochromes and cytochrome *a* respectively. This latter observation suggests that chloramphenicol may have some activity in the terminal oxidase region of the respiratory chain. The trough at 547 nm, due to the oxidation of cytochrome *c*, and the presence of peaks due to reduced *b* cytochromes, indicate a site of inhibition between the two electron carriers, in the region of the antimycin A site. The very rapid appearance of reduced flavoprotein indicated by the trough confirms the conclusion that there is a sensitive site of inhibition in the flavoprotein region, *i.e.* in the region of phosphorylation Site I.

These conclusions are further substantiated by the measurements of the steady state reduction levels of the electron carriers of turnip mitochondria (Table I). Low concentrations of D-*threo*-chloramphenicol, added to mitochondria oxidising malate, caused a marked reduction of flavoprotein. This carrier became more oxidised if succinate was the substrate agreeing with the proposed site of action at the phosphorylation Site I level. Low concentrations also caused a slight reduction of cytochromes *b* and *a* while cytochrome *c* became more oxidised. Higher concentrations increased the reduction of cytochromes *b* and *a* but little further change in the flavoproteins and the cytochrome *c*. The observed changes in cytochrome *b* were similar using either 560–540 or 560–575 nm as wavelength pairs.

TABLE I

THE EFFECTS OF D-*threo*-CHLORAMPHENICOL ON THE STEADY-STATE REDUCTION LEVELS OF THE REDOX CARRIERS OF TURNIP MITOCHONDRIA

Experimental conditions as described in the text.

Substrate	*Carrier*	*% carrier reduced*		
		0 mM D-*threo*-*Chloramphenicol*	*0.33 mM* D-*threo*-*Chloramphenicol*	*4.3 mM* D-*threo*-*Chloramphenicol*
Malate	Flavoprotein	50	71	68
	Cytochrome *b*	32	45	58
	Cytochrome *c*	30	24	21
	Cytochrome *a*	0	7	26
Succinate	Flavoprotein	23	0	3
	Cytochrome *b*	20	24	57
	Cytochrome *c*	25	22	25
	Cytochrome *a*	0	6	0

The results presented in this paper do not indicate a lack of specificity for puromycin, lincomycin, actinomycin D, mitomycin C, and D-serine at the concentrations normally employed for the inhibition of protein synthesis. Cycloheximide and chloramphenicol inhibit the oxidation of NAD-linked substrates apparently acting at the flavoprotein level of the respiratory chain. Chloramphenicol has two additional sites of action on the electron transport chain. There is a second site of variable sensitivity in the antimycin A-sensitive region, between cytochromes *b* and *c*, and a third site which was not inhibited sufficiently to restrict oxygen uptake, in the region of the terminal oxidase. The evidence presented indicates that in plant mitochondria, as in beef heart mitochondria, the primary action of chloramphenicol is on the electron transport and not on the associated energy-conservation systems.

The results presented above support the conclusion that great care must be taken in the interpretation of data obtained using inhibitors. Control experiments therefore should be designed to use inactive isomers and samples of solvents where possible.

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