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INHIBITION OF SELECTED PLANT SYSTEMS BY STEREOISOMERS OF CHLORAMPHENICOL

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Key Word Index—*Avena sativa*; *Triticum vulgare*; *Hordeum vulgare*; Gramineae; chloramphenicol isomers; non-stereospecific inhibition of protein synthesis.

Abstract—The stereospecificity of chloramphenicol isomers on the inhibition of several plant systems was investigated. L-*Threo*, D-*erythro*, L-*erythro* and the antibiotic D-*threo*-chloramphenicol were effective inhibitors of auxin-induced elongation, ^{14}C-leucine uptake and ^{14}C-leucine incorporation into the protein fraction of coleoptiles from *Avena sativa* and *Triticum vulgare*. The isomers also inhibited *Avena* coleoptile uptake of ^{14}C-α-aminoisobutyric acid and the *de novo* synthesis of α-amylase by aleurone layers from *Hordeum vulgare* seeds. All four compounds inhibited these processes to about the same extent and over a similar high concentration range (5×10^{-4} to 5×10^{-3} M). Bioassay of extracts from *Avena* coleoptiles treated with the non-antibiotic isomers revealed no tissue conversion into D-*threo*-chloramphenicol.

INTRODUCTION

CHLORAMPHENICOL is a specific inhibitor of protein synthesis in a wide variety of bacteria.[1] Of the four possible stereoisomers of chloramphenicol (D-*threo*, L-*threo*, D-*erythro* and L-*erythro*), only the antibiotic, D-*threo*-chloramphenicol, inhibits protein synthesis in bacteria.[2,3]

Several plant systems are also inhibited by chloramphenicol.[4] However, the concentration required for the inhibition of plant systems is several fold greater than those required for bacterial systems. Recently, we demonstrated that the high levels of chloramphenicol required for inhibition of elongation and ^{14}C-leucine incorporation into the protein fraction of *Avena* coleoptiles were not due to a lack of absorption or a rapid inactivation of the inhibitor.[5] This pointed to the possibility that chloramphenicol inhibition in higher plants was nonspecific. There is some evidence for this assumption since root growth,[6] ion uptake,[7,8] ^{14}C- or ^{3}H-amino acid incorporation into the protein fraction,[8,9] and oxidative phosphoryl-

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ation[10] were inhibited by L-*threo*-chloramphenicol to an extent comparable to that produced by D-*threo*-chloramphenicol.

In this paper we report the comparative effect of the four isomers of chloramphenicol on several plant processes, namely auxin-induced elongation, uptake of ^{14}C-α-aminoisobutyric acid, incorporation of ^{14}C-leucine into protein, and *de novo* synthesis of α-amylase.

TABLE 1. INHIBITION OF *Avena* COLEOPTILE ELONGATION BY ISOMERS OF CHLORAMPHENICOL

Concn (mM)	D-*threo*	L-*threo*	D-*erythro*	L-*erythro*
0·5	8	2	2	−1
1·0	49	57	46	40
5·0	74	78	70	68

Coleoptiles were incubated for 24 hr. The data (mean of two experiments) are expressed as % inhibition. The elongation of the control coleoptiles was 6·4 mm. IAA was included at a concentration of 10^{-5} M.

RESULTS

Inhibition of Avena *Coleoptile Elongation*

Inhibition of auxin-induced elongation of *Avena* coleoptiles by the four chloramphenicol isomers is illustrated in Table 1. As previously shown[4] the concentration required for inhibition by the antibiotic, D-*threo*-chloramphenicol, was greater than 5×10^{-4} M.

TABLE 2. INHIBITION OF ELONGATION, ^{14}C-LEUCINE UPTAKE AND ^{14}C-LEUCINE INCORPORATION INTO THE TCA INSOLUBLE FRACTION OF *Avena* COLEOPTILES BY THE ISOMERS OF CHLORAMPHENICOL

Measurement	Control	D-*threo*	L-*threo*	D-*erythro*	L-*erythro*
Elongation	2·2[a]	1·0[b]	1·1[b]	1·1[b]	1·1[b]
% inhibition	—	55	50	50	50
Uptake	9976[a]	4292[b]	3607[b]	5181[b]	5479[b]
% inhibition	—	57	64	48	45
Incorporation	4241[a]	1128[c]	1308[c]	1894[b]	1629[bc]
% inhibition	—	73	69	55	62

Coleoptiles were incubated for 4 hr. Elongation is expressed in mm. ^{14}C-Leucine uptake and incorporation are expressed as cpm per 10 coleoptiles.

The data are the mean of four experiments. Means in the same row with a different superscript are significantly different at $P = 0{\cdot}05$. The concentration of chloramphenicol was 5·0 mM and IAA was included at 10^{-5} M.

At 10^{-3} M and 5×10^{-3} M, L-*threo*, D-*erythro* and L-*erythro*-chloramphenicol inhibited cell elongation to about the same extent as D-*threo*-chloramphenicol. A comparison of the effect of the four chloramphenicol isomers on coleoptile elongation, ^{14}C-leucine uptake, and ^{14}C-leucine incorporation into TCA insoluble fraction is given in Table 2. Although there were

some differences in the activity, all four isomers inhibited ^{14}C-leucine uptake and incorporation into the protein fraction, as well as elongation. As previously noted[4,5] it was difficult to evaluate the relative importance of the inhibition of amino acid uptake and the inhibition of protein synthesis, and no further attempt was made to separate these two factors as was done with D-*threo*-chloramphenicol.[4,5]

The stereospecificity of chloramphenicol inhibtion of amino acid uptake by *Avena* coleoptiles was further established by using ^{14}C-α-aminoisobutyric acid. In the control experiment uptake was linear over the four hour period of the experiment (Fig. 1). The internal concentration exceeded the external concentration within the first hour and the amino acid continued to concentrate against a gradient. At the termination of the experiment,

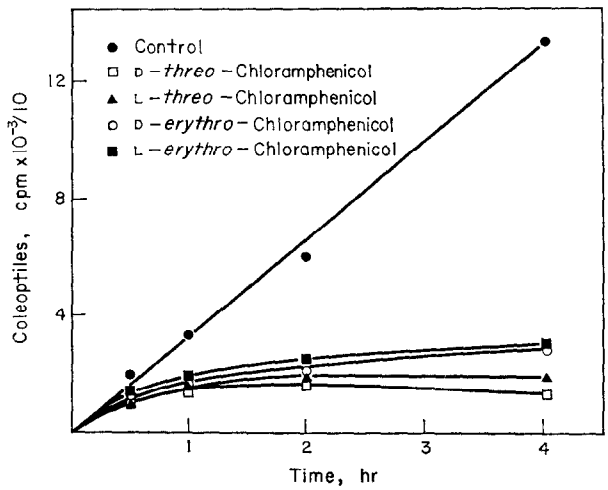

FIG. 1. UPTAKE OF ^{14}C-α-AMINOISOBUTYRIC ACID INTO *Avena* COLEOPTILES: EFFECTS OF THE ISOMERS OF CHLORAMPHENICOL AT 5×10^{-3} M.

the internal concentration exceeded the external concentration by more than 4-fold. The four chloramphenicol isomers markedly inhibited ^{14}C-α-aminoisobutyric acid uptake. The internal concentration did not exceed the external concentration in the treated coleoptiles. ^{14}C-α-Aminoisobutyric acid was not metabolized in this system as no radioactivity was incorporated into the protein fraction and all except minor traces of the 70% ethanol soluble radioactivity chromatographed on two dimensional chromatography[11] with authentic α-aminoisobutyric acid.

Inhibition of Triticum *Coleoptile Elongation*

Chloramphenicol inhibition of auxin-induced elongation of wheat coleoptiles incubated for 22 hr is shown in Table 3. At a concentration of 5×10^{-3} M, all of the isomers inhibited elongation by about 90%. At 10^{-3} M, only L-*threo*-chloramphenicol inhibited elongation. D-*threo*-Chloramphenicol at 5×10^{-4} M produced a marked stimulation of elongation while the non-bacteriostatic isomers (L-*threo*, D-*erythreo* and L-*erythro*) were without effect on coleoptile elongation. When wheat coleoptiles were incubated in an assay solution for

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22 hr, there was a high bacterial population. In those tubes which included D-*threo*-chloramphenicol, no bacterial growth was observed. In those tubes which contained L-*threo*, D-*erythro* or L-*erythro* chloramphenicol, bacterial growth was not inhibited. The stimulation of elongation by 5×10^{-4} M D-*threo*-chloramphenicol was perhaps due to a bacteriostatic action rather than a direct effect on the plant tissue. After 4 hr of incubation, when bacterial contamination was not a problem, 5×10^{-4} M D-*threo*-chloramphenicol did not stimulate elongation (unpublished data).

TABLE 3. INHIBITION OF *Triticum* COLEOPTILE ELONGATION BY THE ISOMERS OF OF CHLORAMPHENICOL

	Isomer				
Concn (mM)	Control	D-*threo*	L-*threo*	D-*erythro*	L-*erythro*
0·5	$7·4^{b}$	$9·2^{a}$	$6·7^{b}$	$6·8^{b}$	$6·8^{b}$
% inhibition	—	−24	9	8	8
1·0	$7·4^{ab}$	$7·8^{a}$	$5·8^{c}$	$6·4^{bc}$	$6·7^{ab}$
% inhibition	—	−5	22	12	9
5·0	$7·4^{a}$	$0·6^{b}$	$0·7^{b}$	$1·0^{b}$	$0·5^{b}$
% inhibition	—	92	91	86	93

Coleoptiles were incubated for 22 hr. Elongation is expressed in mm (mean of three experiments). Means in the same row with a different superscript are significantly different at $P = 0·05$. IAA was included at a concentration of 10^{-5} M.

In Table 4, the effect of the isomers at a concentration of 5×10^{-3} M on elongation, ^{14}C-leucine uptake and incorporation into the protein fraction is presented. All of the isomers inhibited effectively these three processes in the *Triticum* coleoptiles. In the case of

TABLE 4. INHIBITION OF ELONGATION, ^{14}C-LEUCINE UPTAKE, AND ^{14}C-LEUCINE INCORPORATION INTO THE TCA INSOLUBLE FRACTION OF *Triticum* COLEOPTILES BY ISOMERS OF CHLORAMPHENICOL

Measurement	Control	D-*threo*	L-*threo*	D-*erythro*	L-*erythro*
Elongation	2·6	0·9	0·8	1·3	1·1
% inhibition	—	65	69	50	58
Uptake	10760^{a}	4164^{c}	3480^{c}	5673^{b}	4594^{bc}
% inhibition	—	61	68	47	57
Incorporation	3123^{a}	696^{c}	760^{bc}	927^{bc}	1134^{b}
% inhibition	—	78	76	70	64

The incubations were for 4 hr. Elongation is expressed in mm (mean of two experiments). ^{14}C-Leucine uptake and incorporation are expressed as cpm per 10 coleoptiles (mean of four experiments). Means in the same row with a different superscript are significantly different at $P = 0·05$. Chloramphenicol isomers were included at a concentration of 5·0 mM, and IAA at 10^{-5} M.

elongation, the most effective isomer (L-*threo*-) inhibited 69%, while the least active (D-*erythro*-) inhibited 50%. As in the *Avena* coleoptiles, ^{14}C-leucine uptake was also inhibited by the four isomers. The inhibition of ^{14}C-leucine incorporation into the TCA insoluble fraction ranged from 78% for D-*threo* to 64% for L-*erythro*-chloramphenicol.

Inhibition of α-Amylase Synthesis by Barley (Hordeum vulgare) *Aleurone Layers*

On the basis of the above studies, it appeared that chloramphenicol repression of protein synthesis was not stereospecific in plant systems. Thus, a more direct measure of protein synthesis in a plant system where inhibition of amino acid uptake would not confound the results was used. Varner[12] reported previously that D-*threo*-chloramphenicol inhibited the gibberellic acid controlled increase in α-amylase activity of barley endosperm. Varner *et al.*[13–15] also demonstrated that the appearance of α-amylase activity in both half-seeds and aleurone layers was due to *de novo* synthesis of the enzyme. Aleurone layers were incubated with the appropriate buffer containing 10^{-5} M gibberellic acid. When included, the isomers of chloramphenicol were at a concentration of 5×10^{-3} M. After 24 hr of incubation, α-amylase in the medium and in the tissue was examined separately. All of the isomers were effective inhibitors of α-amylase synthesis (Table 5). The inhibition of α-amylase synthesis ranged from 73% with the D-*erythro* isomer to 83% with the L-*threo* compound. In the presence of the inhibitors, the proportion of α-amylase retained in the tissue was greater than in the control.

TABLE 5. INHIBITION OF α-AMYLASE SYNTHESIS IN BARLEY ALEURONE LAYERS BY CHLORAMPHENICOL ISOMERS

Fraction assayed	Control	D-*threo*	L-*threo*	D-*erythro*	L-*erythro*
Medium	12 720	1875	1670	2400	2425
Extract	8460	2675	1840	3400	2850
Total	21 180	4550	3510	5800	5275
% Inhibition of total	0	79	83	73	75

Incubation were for 24 hr. Data (mean of two experiments) are expressed as μg of starch hydrolyzed per min per 10 aleurone layers. Chloramphenicol isomers were included at a concentration of 5·0 mM. All treatments included gibberellic acid at 10^{-5} M.

Assay of Chloramphenicol Isomers after Incubation and Extraction from Avena *Coleoptiles*

The possibility existed that the plant tissue had the capacity to convert L-*threo*, D-*erythro* and L-*erythro* isomers into D-*threo*-chloramphenicol and only the latter isomer was active *per se*. Therefore, possible conversion of the non-antibiotic isomers into D-*threo*-chloramphenicol in the tissue was investigated by determining the ability of extracts from treated

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coleoptiles to inhibit protein synthesis in a strain of *E. coli* which was both sensitive and specific for D-*threo*-chloramphenicol. Extracts sufficient in quantity to give a final inhibitor concentration of approximately 24 μg/ml were added to the *E. coli* cell suspensions and ^{14}C-leucine incorporation into the TCA insoluble fraction was compared to that obtained with an equal extract from control coleoptiles or 25 μg/ml authentic D-*threo*-chloramphenicol (Table 6). Only the acetone extract from the D-*threo*-chloramphenicol treated coleoptiles terminated protein synthesis indicating that conversion of the other isomers into D-*threo*-chloramphenicol did not take place.

TABLE 6. EFFECT OF EXTRACTS OF CHLORAMPHENICOL ISOMER-TREATED *Avena* COLEOPTILES ON ^{14}C-LEUCINE INCORPORATION INTO THE TCA INSOLUBLE FRACTION OF *Escherichia coli*

Direct addition of D-*threo* (25 μg)	Extract of coleoptiles treated with:				
	Untreated	D-*threo*	L-*threo*	D-*erythro*	L-*erythro*
0[b]	326[a]	8[b]	292[a]	350[a]	273[a]

DISCUSSION

Chloramphenicol inhibition of auxin-induced elongation of *Avena* and *Triticum* coleoptiles, ^{14}C-α-aminoisobutyric acid uptake into *Avena* coleoptiles, ^{14}C-leucine uptake and incorporation into the TCA insoluble fraction of both *Avena* and *Triticum* coleoptiles, and αamylase synthesis in barley aleurone layers is not stereospecific. All of the isomers of chloramphenicol inhibited the plant systems studied to about the same degree and required similar high concentrations. This is in sharp contrast to results obtained with bacterial systems which are sensitive to a much lower concentration and where the inhibition is highly specific for D-*threo*-chloramphenicol.[2] These results are similar to those obtained with a cell-free system from Ehrlich ascites tumor.[16] Inhibition of this mammalian system required high levels of chloramphenicol ($2{\cdot}5 \times 10^{-3}$ M) and all four isomers showed comparable degrees of inhibition of protein synthesis.

The inhibition of auxin-induced elongation of *Avena* coleoptiles by D-*threo*-chloramphenicol was similar to that reported by Nooden and Thimann.[4] D-*threo*-Chloramphenicol inhibition of cell elongation in *Avena* coleoptiles[4,17] etiolated pea hypocotyl sections,[4,17] green pea stem sections,[18] artichoke slices,[4,17] potato tuber slices,[19] and sunflower hypocotyl sections[20] was in each case interpreted as evidence for a requirement of protein synthesis in auxin-induced cell elongation.

Since both ^{14}C-leucine uptake and incorporation into the protein fraction of *Avena* and *Triticum* coleoptiles were inhibited by all four isomers of chloramphenicol (Tables 2 and 4), it was difficult to establish the relationship of inhibition of protein synthesis and amino acid uptake with ^{14}C-leucine. An attempt to study independently these two processes

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was made by following the uptake of ^{14}C-α-aminoisobutyric acid (an amino acid not incorporated into protein) by *Avena* coleoptiles, and protein synthesis in barley aleurone layers (in which the consequences of amino acid uptake are minimal). The uptake of ^{14}C-α-aminoisobutyric acid was inhibited by the isomers of chloramphenicol (Fig. 1). In the control tissue, the uptake of the amino acid appeared to be an active process as it was accumulated against a concentration gradient. In the presence of the isomers of chloramphenicol, the internal concentration never exceeded the external concentration.

Varner and coworkers[12–15] showed previously that the *de novo* synthesis of α-amylase during incubation of half-seeds or aleurone layers was sensitive to several inhibitors of protein synthesis including D-*threo*-chloramphenicol. The present work establishes that chloramphenicol inhibition of α-amylase synthesis (Table 5) is not stereospecific.

One explanation for similar activities of the four isomers would be that plant tissue could convert the isomers of D-*threo*-chloramphenicol into the antibiotic. If for example the hydroxyl group adjacent to the benzene ring could be reversibly oxidized and the resulting ketone reversibly enolyzed, the tissue could effect a complete interconversion of the four isomers. With *Avena* coleoptiles, the degree of inhibition by the four isomers at a given concentration was nearly identical. Thus, if the isomers of D-*threo*-chloramphenicol were not active *per se*, there would have to be a near quantitative conversion into the active isomer. When acetone extracts of the treated coleoptiles were assayed for D-*threo*-chloramphenicol by measuring the inhibition of protein synthesis in *Escherichia coli*, no apparent conversion of the non-antibiotic isomers into the antibiotic was detected (Table 6). These experiments demonstrated quite conclusively that to obtain inhibition there was not an obligatory requirement for the plant tissue to convert the L-*threo*, D-*erythro* and L-*erythro* isomers into 'active' D-*threo*-chloramphenicol.

It is unknown whether chloramphenicol acts directly or indirectly on protein synthesis. In bacteria, D-*threo*-chloramphenicol stereospecifically binds to the 50*S* subunit of the 70*S* ribosome[21] and prevents the growth of the peptide on the ribosome. D-*threo*-Chloramphenicol at a low concentration ($3{\cdot}23 \times 10^{-6}$ M) did not bind to 80*S* ribosomes from pea seedlings.[22] Perhaps at higher concentrations, chloramphenicol may bind in a non-stereospecific manner to plant 80*S* ribosomes. This might also be the case in the mammalian cell-free system (80*S* ribosomes) where the L-*threo*, D-*erythro* and L-*erythro* isomers at $2{\cdot}5 \times 10^{-3}$ M were equally as inhibitory as D-*threo*-chloramphenicol.[16] It should be noted that a chloroplast preparation (70*S* ribosomes) from tobacco leaves was sensitive to D-*threo*-chloramphenicol (73% inhibition at $9{\cdot}3 \times 10^{-4}$ M) and this inhibition was stereospecific.[23]

An indirect method of inhibition was proposed by Hanson *et al.*[24,25] These workers found that D-*threo*-chloramphenicol was an effective inhibitor of energy-linked processes in maize mitochondria and suggested that the repression of protein synthesis in plants might be due to the inhibition of ATP synthesis. More recently, Hanson and Krueger[9] reported that L-*threo*-chloramphenicol impaired oxidative phosphorylation more effectively than the D-*threo* isomer. Establishing the exact mechanism of the non-stereospecific inhibition of protein synthesis in plant tissue will require further investigation.

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[23] R. J. Ellis, *Science* **158,** 477 (1969).
[24] J. B. Hanson and T. K. Hodges, *Nature, Lond.* **200,** 1009 (1963).
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EXPERIMENTAL

Coleoptiles from *Avena sativa* (cv. Torch) were obtained as described by Schlender *et al.*[5] *Triticum vulgare* (cv. Thatcher) seeds were soaked in tap H_2O in the dark at 26° for 2 hr. All operations were performed under a green safe-light.[26] The seeds were germinated in moist vermiculite in darkness at 26°. After 70 hr, when the coleoptiles were 2·5–3·5 cm in length, a 4·5 mm section was removed about 3–4 mm below the tip. The sections were floated on H_2O for 1 hr before use. The procedures for study of coleoptile elongation, uptake of ^{14}C-α-aminoisobutyric acid, and uptake and incorporation of ^{14}C-leucine into the TCA insoluble fraction were as previously described.[5,27]

Barley (*Hordeum vulgare* cv. Himalaya) aleurone layers were prepared[15] and, aseptically, 10 layers were incubated in 2 ml of 10^{-5} M gibberellic acid, 10^{-3} M acetate buffer (pH 4·8), and 10^{-2} M $CaCl_2$ along with the appropriate chemical treatment. After 24 hr incubation in the dark at 22°, the medium was decanted and the layers rinsed with 3 ml of the acetate buffer. The aleurone layers were ground in 5 ml of 0·2 M NaCl. After centrifugation at 1000 *g* for 10 min, the supernatant and the medium were assayed for α-amylase activity.[28] The isomers of chloramphenicol at the maximum concentration found in any α-amylase assay (35 μg/ml) did not affect the activity of the enzyme.

For the study of possible racemization, the isomers of chloramphenicol (5×10^{-3} M) were incubated with *Avena* coleoptiles for 4 hr under conditions previously mentioned.[5] (The internal and external concentrations were equal, as determined from quantitative experiments with ^{14}C-D-*threo*-chloramphenicol, and each coleoptile thus contained about 8 μg of chloramphenicol.) 9 treated coleoptiles were extracted with 3 ml of acetone in the dark at 4° for 20 hr.[5] Control coleoptiles were extracted in the same way. The extract was divided into three, acetone was removed *in vacuo* at room temp., and the residues were used directly for the bioassay of D-*threo*-chloramphenicol. The latter compound was assayed by measuring the inhibition of ^{14}C-leucine incorporation into the hot TCA insoluble fraction of a cell suspension of *Escherichia coli*. *E. coli*, Crooks strain, was grown in 10 g tryptone, 10 g yeast extract, 5 g K_2HPO_4, and 10 g glucose in 1 l. H_2O at 35°. After 3–4 hr, when the cells were in log growth phase, 1 ml of the cell suspension was used for the test. The cells were incubated with the extracts for 30 min at 35°, then ^{14}C-leucine (80 000 dpm, sp. act. 250 μCi/μmol) was added and the incubation continued for 60 min. After addition of 2 ml of 10% TCA, the mixture was heated at 80° for 10 min and the insoluble protein filtered off, washed $2 \times 5\%$ TCA, with EtOH–Et_2O (1:1, v/v), and Et_2O. The filter disk was dried and counted in 5 ml of scintillation fluid.[29] This provided a sensitive (over 90% inhibition with 10 μg/ml D-*threo*-chloramphenicol) and stereospecific assay for D-*threo*-chloramphenicol.

When appropriate, the results were summarized by analysis of variance. Differences among treatment means were delineated by Tukey's ω-procedure.

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