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UPTAKE AND METABOLISM OF ^{14}C-CHLORAMPHENICOL BY *AVENA* COLEOPTILES

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Abstract—The uptake and metabolism of ^{14}C-chloramphenicol by coleoptiles of *Avena sativa* (cv. Torch) were related to chloramphenicol inhibition of cell elongation and protein synthesis. While the rate of uptake may account for the lag period before the inhibition of cell elongation and protein synthesis, it is not the reason for the high concentration of chloramphenicol required to inhibit these processes. The antibiotic was not rapidly metabolized by the tissue.

INTRODUCTION

CHLORAMPHENICOL, a specific inhibitor of protein synthesis in bacteria,[1] also inhibits enzyme synthesis and ^{14}C-amino acid incorporation into the proteins of higher plants.[2] The concentration of chloramphenicol required to significantly reduce protein synthesis in excised plant tissue is usually 100–1000 times greater than that required in bacteria.[1,2] Since the basic mechanism of protein synthesis is similar in both bacteria and higher plants, the basis for the difference in sensitivity to chloramphenicol is not clear. Several possibilities have been considered. Firstly, the resistant systems may not absorb chloramphenicol or may absorb it slowly. Vazquez[3] demonstrated a correlation between the inhibitory activity of chloramphenicol and its absorption in several bacteria. In *Nitella clavata*, chloramphenicol uptake was shown to occur by diffusion.[4] The internal concentration was only about 50 per cent of the external concentration after 4 hr incubation and about 80 per cent after 6 hr incubation. This led Nooden and Thimann[2] to speculate that the high concentration of chloramphenicol required to inhibit auxin-induced growth and protein synthesis in *Avena* coleoptiles, pea stem sections, and artichoke tuber disks was due to slow penetration.

Another factor which could affect the sensitivity is rapid metabolism or inactivation of the antibiotic. Certain strains of bacteria produce an extracellular substance capable of inactivating chloramphenicol. In two recent independent studies,[5,6] it was reported that a chloramphenicol-resistant strain of *Escherichia coli* inactivated chloramphenicol by enzymatic acetylation. Cell extracts in the presence of acetyl-coA and chloramphenicol catalyzed the

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formation of 3-acetoxy- and 1,3-diacetoxychloramphenicol. In studies with a variety of animals, including man, most of an orally administered dose of chloramphenicol was recovered in the urine within 24 hr as a glucuronic acid conjugate.[7]

A third consideration is a differential binding of chloramphenicol to the ribosomes of sensitive and non-sensitive organisms. Studies on the binding of chloramphenicol to the ribosomes of a number of organisms, including etiolated pea ribosomes, revealed that chloramphenicol was bound to the ribosomes of sensitive organisms, but not to the ribosomes of resistant organisms.[3]

In this paper, we report the uptake and metabolism of ^{14}C-chloramphenicol in *Avena* coleoptiles. In addition, the kinetics of chloramphenicol uptake are compared to chloramphenicol inhibition of cell elongation and protein synthesis.

RESULTS AND DISCUSSION

Chloramphenicol Inhibition of Cell Elongation and Protein Synthesis

Chloramphenicol inhibition of cell elongation was established in the presence of 10^{-5} M indole-3-acetic acid (IAA). After 2 hr, elongation of coleoptiles treated with 10^{-5} M IAA and 10^{-3} M chloramphenicol was inhibited (Fig. 1). The elongation from 4 to 24 hr continued at a linear rate, but the rate was appreciably reduced as compared to the IAA controls. Inhibition was obtained within 2 hr when a concentration of 5×10^{-3} M chloramphenicol was used in the presence of 10^{-5} M IAA (Fig. 1). Thereafter, the growth rate declined steadily

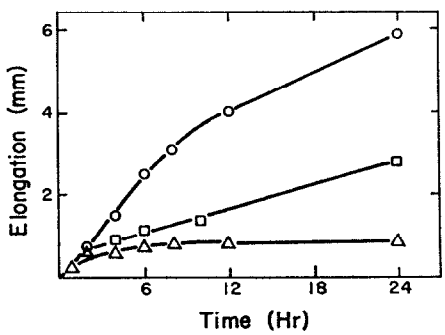

FIG. 1. TIME COURSE OF CHLORAMPHENICOL INHIBITION OF THE ELONGATION OF *Avena* COLEOPTILES. The treatments were: 10^{-5} M IAA ○——○, 10^{-5} M IAA plus 10^{-3} M chloramphenicol □——□, and 10^{-5} M IAA plus 5×10^{-3} M chloramphenicol △——△. Each point is the mean of three experiments.

until after 6 hr when the inhibition was complete. In addition, pretreatment of the tissue with a solution of 5×10^{-3} M chloramphenicol for 45 min or longer before the addition of IAA prevented auxin-induced elongation.

The time course of ^{14}C-leucine uptake and incorporation into the trichloroacetic acid insoluble fractions is shown in Table 1. ^{14}C-Leucine uptake and incorporation into the trichloroacetic acid fraction were both inhibited by chloramphenicol (5×10^{-3} M) within 30 min, and both proceeded at a much reduced rate compared with the IAA controls throughout the 6-hr duration of the experiment. As in previous experiments with plant systems,[8] it was difficult to assess the relative importance of the inhibition of amino acid uptake and the direct

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inhibition of protein synthesis. However, when coleoptiles were pretreated with ^{14}C-leucine and then transferred to IAA and IAA plus 5×10^{-3} M chloramphenicol, protein synthesis in the presence of chloramphenicol was inhibited over a 2-hr period (Table 2). This confirms that protein synthesis was inhibited directly and not through inhibition of amino acid uptake. These experimental results confirm those reported by Nooden and Thimann.[2]

TABLE 1. UPTAKE AND INCORPORATION OF ^{14}C-LEUCINE INTO THE PROTEIN OF *Avena* COLEOPTILES TREATED WITH 5×10^{-3} M CHLORAMPHENICOL

Time (hr)	Uptake* IAA control	Uptake* Chloramphenicol	Uptake* % Inhibition	Trichloroacetic acid† insoluble fraction IAA control	Trichloroacetic acid† insoluble fraction Chloramphenicol	Trichloroacetic acid† insoluble fraction % Inhibition
0·5	3,241	1,965	39	820	510	38
1	7,209	3,253	55	1,754	1,028	41
2	14,981	5,036	66	4,100	1,828	55
3	19,486	6,357	67	6,824	1,602	77
4	23,608	6,770	71	9,547	2,146	78
6	33,136	7,024	78	12,241	2,450	78

* Total uptake was obtained as described in the text and expressed as cpm/10 coleoptiles. The data are the mean of five experiments.

† The trichloroacetic acid insoluble fraction was obtained as described by Key[20] and expressed as cpm/10 coleoptiles. The data are the mean of five experiments.

TABLE 2. CHLORAMPHENICOL (5×10^{-3} M) INHIBITION OF ^{14}C-LEUCINE INCORPORATION INTO THE PROTEIN OF *Avena* COLEOPTILES PRETREATED WITH ^{14}C-LEUCINE*

	Initial	2 hr IAA control	2 hr Chloramphenicol
Total	5662[a]	5725[a]	5170[a]
Trichloroacetic acid insoluble	1543[a]	2200[b]	1818[a]

* *Avena* coleoptiles were pretreated with ^{14}C-leucine for 2 hr. One group of coleoptiles was used to determine the initial total uptake and incorporation into the trichloroacetic acid fraction. The other coleoptiles were transferred to either 10^{-5} M IAA or 10^{-5} M IAA plus 5×10^{-3} M chloramphenicol and incubated for an additional 2 hr. The data, expressed as cpm/10 coleoptiles, are the mean of six experiments. Means in the same row with different superscripts differ significantly at $P = 0{\cdot}05$.

Chloramphenicol Uptake and Metabolism

The uptake of ^{14}C-chloramphenicol at 10^{-3} and 5×10^{-3} M concentration was such that the internal approached the external concentration in both treatments within 4-hr (Fig. 2). The entry into the tissue was probably by simple diffusion as reported for *Nitella*.[4] Although penetration may explain the lag period before chloramphenicol inhibition of cell elongation and ^{14}C-leucine incorporation into protein, it was not the reason for the high concentration required for inhibition (threshold of inhibition greater than 10^{-4} M). When a 5×10^{-3} M solution of ^{14}C-chloramphenicol was employed, the internal concentration exceeded 10^{-3} M within 30 min.

The metabolism of ^{14}C-chloramphenicol was studied by TLC of the acetone extract of coleoptiles which had been incubated for 4 hr in a 5×10^{-3} M solution of the inhibitor. Using $CHCl_3$/ethyl acetate/formic acid (5:4:1), approximately 90 per cent of the radioactivity coincided with authentic chloramphenicol (Fig. 3A). In $CHCl_3$/benzene/ethanol (7:3:1), over 80 per cent of the radioactivity chromatographed as chloramphenicol (Fig. 3B).

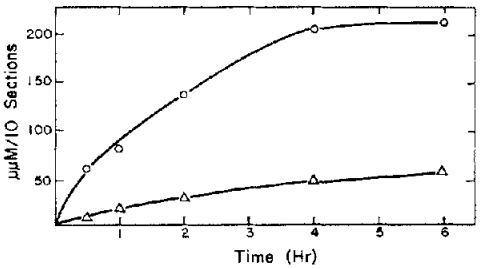

FIG. 2. TIME COURSE OF ^{14}C-CHLORAMPHENICOL UPTAKE BY *Avena* COLEOPTILES.
The treatments were: 10^{-3} M ^{14}C-chloramphenicol △——△ and 5×10^{-3} M ^{14}C-chloramphenicol ○——○. Each point is the mean of two experiments.

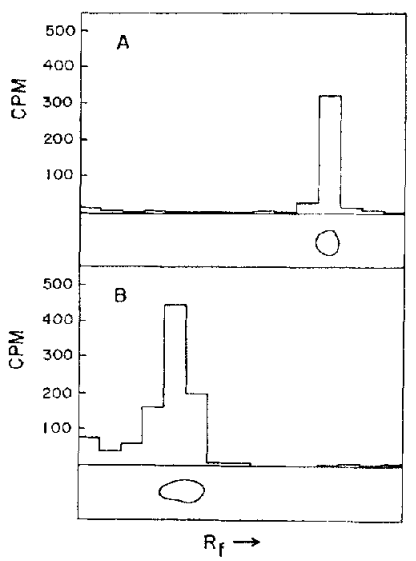

FIG. 3. TLC OF AN ACETONE EXTRACT OF *Avena* COLEOPTILES INCUBATED FOR 4 hr WITH 5×10^{-3} M ^{14}C-CHLORAMPHENICOL.
Solvent A: $CHCl_3$/ethyl acetate/formic acid (5:4:1, v/v). B: $CHCl_3$/benzene/ethanol (7:3:1, v/v).

There was no chromatographic evidence for the formation of either 3-acetoxychloramphenicol or 1,3-diacetoxychloramphenicol, both of which were run as standard markers. In addition, the extract inhibited both the growth and ^{14}C-leucine incorporation into the protein fraction of *Escherichia coli*.

By comparison of the time course of chloramphenicol inhibition of cell elongation and protein synthesis to that of chloramphenicol uptake, it is evident that the high concentration required for inhibition cannot be explained by a lack of absorption or a slow rate of penetration of the antibiotic. In addition, a rapid inactivation of the inhibitor was not an important factor in the *Avena* coleoptile being so insensitive to chloramphenicol.

The inhibitory effect of chloramphenicol on sensitive systems appears to be associated with the binding of the inhibitor to the ribosomes.[3] Vazquez[9] demonstrated that when chloramphenicol is taken up by a number of bacteria, the antibiotic was bound to the 50S subunit of the 70S ribosome. He also found that chloramphenicol was not bound to the 80S ribosomes of yeast, rat liver, etiolated pea seedlings and protozoa, all organisms which are resistant to low levels of chloramphenicol.[1] In the *Avena* coleoptile, which like other plants contain 80S ribosomes in the cytoplasm,[10] the resistance may be related to a low degree of binding to the ribosomes.

It is of interest that plant chloroplasts, which contain ribosomes similar to the 70S ribosomes of bacteria,[1] are sensitive to low concentrations of chloramphenicol.[11] Anderson and Smillie[11] have shown a preferential binding of ^{14}C-chloramphenicol to the chloroplast ribosomes as compared to cytoplasmic ribosomes of pea and wheat. Also, protein synthesis by mitochondria isolated from yeast, mammalian and plant tissue[12–14] was sensitive to low concentrations of chloramphenicol. This may contribute to the reported chloramphenicol inhibition of oxidative phosphorylation of plant mitochondria.[15,16] However, the latter inhibition required a much higher concentration of chloramphenicol.

EXPERIMENTAL

Avena sativa (cv. Torch) seeds were soaked in tap water at 26° for 2–3 hr. The seeds were then spread evenly on moist vermiculite in glass trays and germinated in a growth room under a dim red light at 26° and 85–95 per cent relative humidity. After 24 hr the seeds were covered with a thin layer of vermiculite and incubated in the darkened growth room. All further operations were carried out under a green-safe light. About 70 hr after planting when the coleoptiles were 2–3 cm long, 4·5-mm sections were cut 2–3 mm below the tip. These were floated for 2 hr on a glass-distilled water solution containing 1 mg of $MnSO_4 \cdot H_2O$ per l.[17]

For the study of the uptake of ^{14}C-chloramphenicol, incubation of the coleoptiles was carried out in the dark at 26° in citrate–phosphate buffer (pH 5·0) containing 2% sucrose and 0·1% Tween 80.[17] Ten coleoptiles were placed in each test tube with 1 ml of the designated ^{14}C-chloramphenicol solution and incubated on a revolving drum (1 rev/min). After the incubation period, the coleoptiles were placed on a wire screen, rinsed with distilled water and transferred to a 10 ml beaker. The coleoptiles were rinsed for 1 min in 8 ml of distilled water, blotted dry, and then transferred to a scintillation vial containing 15 ml of scintillation fluid.[18] After equilibration for 8 hr at 4° the radioactivity was determined. Disruption of the tissue in the scintillation fluid by sonication did not improve the counting efficiency over that of the intact coleoptiles.

For the investigation of chloramphenicol metabolism, *Avena* coleoptiles were incubated with 5×10^{-3} M ^{14}C-chloramphenicol (3684 cpm/μmole) for 4 hr. The coleoptiles were rinsed, blotted, and transferred to a 5-ml vial containing 3 ml of acetone. The vial was capped and placed in the dark at 4°. After a 5-hr extraction, the coleoptiles contained less than 3% of the initial activity. The acetone extract was evaporated to dryness under N_2 and the residue was dissolved in a small volume of acetone for chromatography.

Chloramphenicol and its acetoxy derivatives were chromatographed on thin-layer silica gel sheets (Eastman Chromatogram Type K301-R2) using $CHCl_3$/benzene/ethanol (7:3:1, v/v) and $CHCl_3$/ethyl acetate/formic acid (5:4:1, v/v). Nonlabeled chloramphenicol and its 3-acetoxy and 1,3-diacetoxy derivatives were detected by spraying with a solution of 0·05% Rhodamine B in ethanol and viewing in u.v. light (258 nm).

^{14}C-Chloramphenicol extracts were chromatographed on 4×20 cm thin-layer sheets. A nonlabeled chloramphenicol marker was spotted on the left lane and the radioactive extract on the right lane. The chromatogram was developed for 15 cm. After drying, the chromatogram was cut to separate the marker

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lane from that of the extract. For location of the radioactive metabolic products the chromatogram was cut into fifteen equal segments. The radioactivity of each segment was determined by liquid scintillation spectrometry.

The time course of chloramphenicol inhibition of auxin-induced elongation was determined in the same buffer in the presence of 10^{-5} M IAA. The procedure was the same as previously described.[19] The uptake and incorporation of ^{14}C-leucine into the trichloroacetic acid insoluble fraction was studied using the same incubation system. Fifteen coleoptiles were used in each experiment. After the indicated incubation, five coleoptiles were selected at random for determination of total uptake of ^{14}C-leucine. They were treated as described for the uptake of ^{14}C-chloramphenicol with the exception that the coleoptiles were disintegrated directly in the scintillation fluid by sonication (Branson Sonic Power Sonifier). The remaining ten coleoptiles were used to determine the incorporation of ^{14}C-leucine into the trichloroacetic acid insoluble fraction. The procedure for the trichloroacetic acid precipitation of the protein fraction was as described by Key.[20] The radioactivity of the fraction was determined by liquid scintillation spectrometry.

D-Threo-chloramphenicol, 3-acetoxychloramphenicol, and 1,3-diacetoxychloramphenicol were kindly supplied by Dr. M. Rebstock, Park Davis and Company. Uniformly labeled ^{14}C-leucine (250 μc/μmole) and dichloroacetyl labeled ^{14}C-D-threo-chloramphenicol (3 04 μc/μmole) were obtained from New England Nuclear Corporation.

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