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PHYSIOLOGICAL AND BIOCHEMICAL STUDIES ON PICLORAM ACTION IN PLANTS

BY

MAHENDRA P. SHARMA

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled, "Physiological and Biochemical Studies on Picloram Action in Plants" submitted by Mahendra P. Sharma in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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Date July 29, 1971

ABSTRACT

The metabolic effects of 4-amino-3,5,6-trichloropicolinic acid (picloram) together with its uptake, translocation, and degradation were studied in soybean (*Glycine max* (L.) Merr. cv 'Harosoy 63') (extremely sensitive to picloram), Canada thistle (*Cirsium arvense* (L.) Scop.) (sensitive), and barley (*Hordeum vulgare* (L.) cv 'Parkland') or corn (*Zea mays* (L.) cv 'Morden 77') (resistant).

Photosynthetic $^{14}CO_2$-fixation by excised leaves of soybean and Canada thistle was reduced significantly by prior exposure of the roots of source plants to picloram. In corn little or no inhibition occurred in fixation. The treatments had no significant effect on $^{14}CO_2$ fixation in the dark. Treatments which caused 50 per cent or more inhibition of fixation resulted in nearly 50 per cent reduction in labeled sucrose and alanine in the leaves. Relative amounts of labeled malic acid, aspartic acid, glutamic acid, asparagine, and serine, on the other hand, increased. Translocation of ^{14}C-assimilates from leaves to roots in intact soybean and Canada thistle plants was inhibited if roots were treated with picloram before exposure to $^{14}CO_2$. Transport of ^{14}C-assimilates in corn plants was not significantly affected by picloram treatment.

Foliar application of picloram reduced the chlorophyll content of soybean and Canada thistle plants. RNA and protein contents of such picloram-treated plants were increased up to 30 per cent over controls. In barley, on the other hand, picloram showed very little effect on chlorophyll, RNA, and protein contents.

In excised sections of soybean hypocotyl, barley coleoptile, barley leaf, and Canada thistle leaf, picloram at 10 µg/ml promoted growth (increase in fresh weight) and enhanced RNA and protein biosynthesis (incorporation of ^{14}C-8-ATP and ^{14}C-U-leucine), whereas at 500 µg/ml, it inhibited growth and RNA and protein biosynthesis. Incorporation of radioactivity into RNA and protein of particulate and supernatant fractions appeared to be equally sensitive to picloram. Actinomycin D and cycloheximide markedly inhibited both normal and picloram-induced growth and RNA and protein biosynthesis. Puromycin was less effective in inhibiting growth and protein biosynthesis. Picloram, GA, and IAA all promoted growth and RNA and protein biosynthesis though they did so at different concentrations, and to varying degrees.

Autoradiography and radioassay results indicated that ^{14}C-picloram was absorbed and translocated rapidly in Canada thistle, soybean, and barley plants following foliar or root application. Foliar absorption was much faster and more complete in soybean and Canada thistle than in barley. The radioactivity from ^{14}C-picloram accumulated in shoot meristems in soybean and Canada thistle, whereas in barley it was more or less evenly distributed throughout the plant following uptake by the foliage or roots.

Uptake of ^{14}C-picloram by excised soybean hypocotyls, barley coleoptiles, barley leaf sections, and Canada thistle leaf discs increased with increase in temperature and was promoted by added ATP and sucrose but inhibited by actinomycin D, cycloheximide, and DNP.

Decarboxylation of ^{14}C-picloram by foliarly-treated Canada thistle, soybean, and barley plants did not occur in appreciable amounts. Chromatographic analysis of ethanol extracts of plants treated with ^{14}C-picloram up to 20 days revealed no evidence of picloram metabolism by these plant species. In excised barley and Canada thistle leaf tissues, on the other hand, 3 days after treatment, ^{14}C-picloram was found conjugated with plant constituents, largely with sugars. After acid hydrolysis of ethanol extracts of ^{14}C-picloram treated excised leaf tissues only unaltered picloram was detected.

Differences in absorption and in distribution patterns of picloram after shoot or root application, together with differential effects, direct or indirect, on nucleic acid and protein metabolism, photosynthesis, and photosynthate translocation are important factors responsible for susceptibility or resistance of the species studied to this herbicide.

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ATP	adenosine 5' triphosphate
Ci	curie
Cpm	counts per minute
DNA	deoxyribonucleic acid
DNP	dinitrophenol
D-RNA	DNA-like RNA
Dpm	disintegrations per minute
GA	gibberellic acid
IAA	indoleacetic acid
LSD	least significant difference
RNA	ribonucleic acid
mRNA	messenger RNA
rRNA	ribosomal RNA
tRNA	transfer RNA
sRNA	soluble RNA
Tris	tris (hydroxymethyl) aminomethane
U	uniformly labeled radioisotope
v/v	volume/volume

INTRODUCTION

Picloram (4-amino-3,5,6-trichloropicolinic acid), a fairly recent introduction into the herbicidal field, is one of the most potent plant growth regulators known. Although picloram is structurally different from well-known auxins such as the phenoxyacetic acid derivatives, it appears to function as a growth regulator of the auxin type (52, 60, 79, 100). Picloram is highly phytotoxic to broadleaf plants while grasses exhibit various degrees of tolerance (34, 63). It is particularly effective on many hard-to-kill deep-rooted perennial weeds (4, 91, 162, 169).

The mechanism(s) through which picloram exerts its control over plant growth have not been fully elucidated. There is little convincing evidence with respect to its possible mode(s) of action and herbicidal selectivity.

The research reported in this thesis was undertaken in order to learn more about the physiological and biochemical responses of Canada thistle (*Cirsium arvense* (L.) Scop.) (sensitive to picloram), soybean (*Glycine max* (L.) Merr., cultivar Harosoy 63) (extremely sensitive), barley (*Hordeum vulgare* L., cultivar Parkland) and corn (*Zea mays* L., cultivar Morden 77) (resistant). The metabolic processes studied included photosynthesis, translocation of photosynthate, and RNA and protein metabolism. In addition, uptake, translocation and degradation of picloram in these plant species were studied.

LITERATURE REVIEW

A. 4-amino-3,5,6-trichloropicolinic acid

The herbicidal properties of picloram (4-amino-3,5,6-trichloropicolinic acid) were first reported in 1963 by Hamaker *et al.* (63). Picloram has shown more toxicity to many broadleaf plants than 2,4-D ((2,4-dichlorophenoxy)acetic acid) and 2,4,5-T ((2,4,5-trichlorophenoxy)acetic acid), but is only moderately toxic to grasses (63). It has effectively controlled deep-rooted perennial weeds such as Canada thistle, field bindweed (*Convolvulus arvensis* L.), and leafy spurge (*Euphorbia esula* L.) (4, 91, 162, 169).

At herbicidal concentrations, picloram causes stem twisting and other formative effects in susceptible dicotyledonous plants. The distortions of growing leaves and stems are similar to those produced by 2,4-D (34, 52, 63, 100). Picloram induced anatomical alterations in leaf tissues of red kidney bean (*Phaseolus vulgaris* L.) (52), Canada thistle (92), and western ironweed (*Vernonia baldwinii* Torr.) (142) and in root tissues of Canada thistle (80, 146). The abnormalities included destruction and distortion of cortical cells and cambial and phloem disintegration.

Picloram has been classified as an auxin-like herbicide similar to 2,4-D and has been shown to cause epinastic responses characteristic of auxins (46, 52). Picloram promoted the growth (fresh weight and length) of etiolated sections of soybean hypocotyl (46), wheat (*Triticum vulgare* L.) coleoptile (79), and oat (*Avena sativa* L.)

coleoptile (79, 140) in a range of concentrations from 1×10^{-6} to 1×10^{-4} M but inhibited their growth at higher concentrations. Goodin and Becher (60) reported that picloram could substitute as an auxin source for callus growth. Qualitatively, picloram, 2,4-D, and IAA cause similar growth responses in tissue sections (46, 79, 102). They all support growth of tissue explants in culture (60), inhibit root growth (78, 100), induce cell wall loosening (38, 45), produce stem curvature and other formative effects, and induce callus growth in girdling tissue (34, 52).

B. Effects of Herbicides on Plant Metabolism

I. Photosynthesis

It is possible that the effects of some herbicides on growth may be linked directly or indirectly to photosynthetic inhibition. As suggested by Sasaki and Kozlowski (137) herbicides may interfere with photosynthesis in several ways such as by (a) clogging or closing stomates, (b) altering optical properties of leaves, (c) altering the heat balance of leaves, (d) affecting metabolism directly, and (e) inducing anatomical changes in tissues involved in photosynthesis. Various interactions among these may also be involved. If herbicides influence the photosynthetic mechanism directly, the rate of CO_2 uptake should be altered rapidly. However, if photosynthesis is influenced indirectly by herbicides, the change in rate will appear gradually.

Evidence from several sources indicates that photosynthesis may be affected by auxins and auxin-herbicides, both directly and

indirectly (30, 101, 137, 172, 175). A concentration of 100 µg/ml IAA and 2,4-D was found by Freeland (56) to reduce the rate of CO_2 uptake by 20 per cent in bean leaves during an experimental period of 4 days. Sprays of 0.001 to 0.1 per cent 2,4-D were used by Loustalot and Muzik (98) in their studies of the effect of the herbicide on velvet bean (*Stizolobium derringianum*) seedlings. Photosynthesis was not affected by the lowest concentration until a week or more after application. The two highest concentrations of 2,4-D (0.05 and 0.1 per cent) produced a sharp drop in the rate of photosynthesis as little as 5 hours after treatment. The higher concentrations of 2,4-D also resulted in damage to mesophyll and to stem phloem. The direct effect of 2,4-D on the photosynthetic rate apparently was augmented indirectly by tissue damage and impaired translocation.

Picloram has been shown to inhibit the photochemical activity (Hill reaction) of isolated chloroplasts of red kidney bean (64). Alterations in the internal anatomy of bean (52), Canada thistle (92) and western ironweed (142) leaves, and partial closure of bean leaf stomata (130) after treatment with picloram at relatively high concentrations suggest that picloram probably affects photosynthetic processes indirectly. At low concentrations, picloram does not seem to have significant effects on photosynthesis. Plengvidhya and Burris (126) reported that picloram did not inhibit photophosphorylation by spinach (*Spinacea oleracea* L.) chloroplasts at concentrations up to 1×10^{-4} M. No information is available as yet on comparative effects of this potent herbicide on $^{14}CO_2$-fixation in picloram-sensitive and -resistant species.

II. Translocation of ^{14}C-Assimilates

There are two major systems in the plant which are involved in translocation, the symplast and the apoplast. These terms were introduced by Münch in 1930, according to Leonard (93), with symplast designating the sum total of the interconnected living protoplast of the plant and the apoplast the non-living cell wall phase around the symplast. The symplast is connected from cell to cell by plasmodesmata connections or tubules. The sieve tubes are considered a part of the symplast, while tracheids, xylem vessels, cell walls, and intercellular spaces are regarded as making up the apoplast.

The upward transport through the xylem in the transpiration stream is considered to be due mainly to the reduced pressure resulting from the evaporation of water from the mesophyll (41). Translocation in the symplast is more complicated. Although several theories have been proposed to explain the mechanism of translocation in this living conduit, no one of them has ever been generally accepted (156). However, there is considerable evidence that the translocation of photosynthetic assimilates occurs in sieve/tubes of phloem of higher plants (160) and follows a source-to-sink pattern, from the regions of synthesis of foods to regions of their utilization (e.g. 6, 19, 76, 157).

Plant growth regulators such as IAA, GA, kinetin, and benzyladenine have been shown to influence the distribution of ^{14}C-assimilates in plants (54, 67, 145). They may result in an increase in retention (54, 145) or a stimulation in translocation (67) of photosynthetic assimilates by the treated parts of the plant.

Evidence has been accumulated to show that phytotoxic dosages of auxin-herbicides such as 2,4-D, 2,4,5-T, and picloram can appreciably alter the translocation or sites of accumulation of photosynthetically assimilated ^{14}C in plants (30, 42, 94, 95, 96, 97). Leonard *et al.* (94) reported that the capacity of red maple (*Acer rubrum* L.) and white ash (*Fraxinus americana* L.) to move labeled assimilates out of their leaves was not destroyed by herbicidal dosages of either 2,4,5-T or amitrole (3-amino-1,2,4-triazole). However, translocation of labeled assimilates to the roots was reduced if the $^{14}CO_2$-treated leaves had been sprayed previously with either herbicide. In the only report available on picloram, Leonard *et al.* (97) observed that both picloram and 2,4-D caused a reduction in translocation of ^{14}C-assimilates into the roots, but enhanced translocation and accumulation by the stems and mature leaves of grape (*Vitis vinifera* L.).

Alterations in the translocation or sites of accumulation of assimilates in plants treated with herbicides may be due to a disruption in the transport system caused by certain herbicides (45, 96, 98). Crafts and Yamaguchi (42) suggested that lack of translocation of labeled amitrole into the hypocotyl of bean plants was due to an effect of 2,4-D, applied 2 days earlier, on the phloem. Though such interference with phloem transport is not critically established, a destruction of sieve tube elements in the phloem of stems by 2,4-D (42, 98) and picloram (52, 61) at higher concentrations and with longer periods of exposure is evident.

III. Chlorophyll Content

A number of reports exist in the literature on the effect of 2,4-D on chlorophyll content of susceptible and resistant plants (101, 174, 175). To my knowledge, no such information is available for picloram. Applications of 2,4-D have been shown by many investigators to reduce substantially the chlorophyll level in treated plants. Williams and Dunn (174) observed that 1 mg/ml 2,4-D caused a decrease in chlorophyll content of 20-day old mustard (*Brassica juncea* L.) plants. They concluded that reduced chlorophyll content, by lowering photosynthetic efficiency of the chloroplasts, was responsible for a reduction in dry weight of the 2,4-D-treated plants. Other studies by Wedding *et al.* (172), in which a decolorization of tissue with increasing amounts of 2,4-D was observed, suggest that the herbicide either interferes with the formation of chlorophyll, or causes increased destruction. It has been suggested by Bruinsma (27) that auxin-herbicides influence the chlorophyll content of plant parts rather more indirectly, mainly by affecting growth or developmental rates.

IV RNA and Protein Metabolism

a. General

Recent reviews have dealt with RNA and protein metabolism in relation to plant growth regulators (66, 83, 109, 159). There is now substantial evidence that auxins and auxin-herbicides have a role, direct or indirect, in nucleic acid and protein metabolism.

Work from the laboratory of Skoog first showed an effect of plant hormones on nucleic acid metabolism. Silberger and Skoog (149) demonstrated an increase in the RNA and DNA content of auxin-treated tobacco pith. Concentrations of IAA which were optimal for cell enlargement and RNA synthesis caused few if any cell divisions in spite of increases in nuclear material. Higher concentrations of auxin blocked both cell enlargement and nucleic acid accumulation. Thus Skoog's was the first suggestion that the action of a plant hormone is intimately concerned with nucleic acid, and consequently with protein metabolism. Since this pioneering study, numerous investigations of the influence of natural and synthetic auxins on RNA and protein metabolism both in intact plants and excised tissues have been reported and will be considered here.

b. Intact Seedlings

Rebstock *et al.* (127) showed that a herbicidal concentration of 2,4-D applied to cranberry bean plants resulted in massive accumulation of nucleic acids and protein. Similar observations on 2,4-D have been made in a variety of plant species such as cocklebur (*Xanthium* sp.) (30), cucumber (*Cucurbita maxima* L.) (173), and soybean (85, 87). The most complete description of nucleic acid synthesis as correlated with aberrant growth in 2,4-D-treated seedlings is that of Key *et al.* (87). Sprays of 2,4-D on soybean seedlings suppressed the synthesis of DNA, RNA, and protein, growth, and cell division in the apical region of the hypocotyl up to 48 hours. On the other hand, more basal tissue swelled, began synthesizing nucleic acids and protein, and by 12

hours had initiated cell division. In 48 hours rRNA content of the hypocotyl trebled, with smaller increases in the soluble and large particle fractions.

Chrispeels and Hanson (36) studied the subcellular distribution of RNA synthesized in hypocotyls of 2,4-D-treated soybean seedlings and found that a lethal concentration of 2,4-D induced the synthesis of massive amounts of RNA. More than half of the increase occurred in the microsomal fraction. The authors postulated that auxin herbicides renewed nuclear activity in the tissue which enhanced synthesis of RNA and protein and tissue proliferation. These relationships have recently been reviewed by Hanson and Slife (66) and implications are that the lethal action of auxin-herbicides relates to their hormonal effects rather than to some direct inhibitory action.

Malhotra and Hanson (100) reported that herbicidal levels of picloram caused increases in the levels of DNA, RNA, and protein in susceptible species (soybean and cucumber) but not in resistant species (barley and wheat). They noted that the increase in RNA was primarily ribosomal. Baur *et al.* (17) have recently shown that a low concentration of picloram significantly increased the soluble protein content of the dicot species cotton (*Gossypium hirsutum* L.) and cowpea (*Vigna sinensis* L.). Monocot species, corn, rice (*Oryza sativa* L.) and wheat, on the other hand, showed a decrease in protein content following picloram treatment.

Evidence provided above suggests that herbicidal levels of synthetic auxins interfere with the normal nucleic acid and protein

metabolism of susceptible species. In apical meristems nucleic acid metabolism and growth are 'frozen'. Basal tissues respond with massive nucleic acid synthesis followed by protein synthesis and cell division. The resistant grasses respond only weakly or not at all, a property associated with high levels of ribonuclease (66, 100).

c. Excised Plant Tissues

Many excised plant tissues exhibit losses in nucleic acid and protein when they are incubated in water or buffer solutions. Such losses have been observed in excised cucumber hypocotyls (173), cotton cotyledons (13), pea stem sections (50), soybean hypocotyls (85), and excised leaves from a number of different plant species (159). Even with this limitation, excised tissues are usually preferred to intact plants for studies of RNA and protein metabolism because of greater manipulative ease in feeding radioactive precursors and in applying various hormones or inhibitors. Numerous studies, recently reviewed by Hanson and Slife (66), Key (83), and Trewavas (159) have shown effects of plant growth regulators on nucleic acid and protein metabolism of excised plant tissues.

Varied responses have been obtained with auxins and auxin-herbicides in RNA and protein metabolism of different plant tissues (Table 1). In elongating corn coleoptiles (81, 173) and in cotton cotyledons (13) auxin (2,4-D) at optimum concentrations for growth enhances the rate of RNA breakdown, while growth inhibitory concentrations prevent this loss in RNA. Results from experiments where the incorporation of ^{14}C-adenosine (81) and ^{14}C-orotic acid (12) was used

TABLE 1

The effect of auxin on RNA and protein levels and on the incorporation of labeled precursors into RNA and protein in various plant tissues

Auxin	Tissue	Precursors	Effect on fr. wt. or length	Effect on Concentration of:		Effect on incorporation of labeled precursors into:		Reference
				RNA	protein	RNA	protein	
	Low concentrations (10-100 μM)							
2,4-D	Cucumber hypocotyl		Increase	Decrease				173
2,4-D	Corn mesocotyl	^{14}C-adenine	Increase	Decrease		Decrease		81,173
2,4-D	Cotton cotyledon	^{14}C-orotic acid	Increase	Decrease		Decrease		12,13
2,4-D	Peanut cotyledon	^{32}Pi				Decrease		31
2,4-D	Soybean hypocotyl	^{14}C-ADP or ATP, ^{32}Pi	Increase	Increase or None		Increase		82,84,85 86,88
2,4-D	Pea stem	^{14}C-glycine	Increase		Increase		Increase	49
Picloram	Soybean hypocotyl	^{32}Pi	Increase	Increase	Decrease	None	Increase	77
IAA	Oat coleoptile	^{32}Pi, ^{3}H-uridine, ^{14}C-leucine	Increase			Increase	Increase	65,105,155
IAA	Pea stem	^{32}Pi, ^{14}C-leucine, ^{14}C-glycine	Increase	Increase	Increase	Increase	Increase	49,158
NAA	Bean endocarp	^{14}C-uridine, ^{14}C-leucine		Increase	Increase	Increase	Increase	134

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TABLE 1 (continued)

Auxin	Tissue	Precursors	Effect on fr. wt. or length	Effect on Concentration of:		Effect on incorporation of labeled precursors into:		Reference
				RNA	protein	RNA	protein	
High concentration (1-4 mM)								
2,4-D	Cucumber hypocotyl		Decrease	Increase	Increase			173
2,4-D	Corn mesocotyl	^{14}C-adenine	Decrease	Increase		Decrease		81,173
2,4-D	Cotton cotyledon	^{14}C-orotic acid		Increase		Decrease		12,13
2,4-D	Peanut cotyledon	^{32}Pi				Decrease		31
2,4-D	Soybean hypocotyl	^{14}C-ADP or ATP, ^{14}C-leucine	Decrease	Decrease or None		Decrease	Decrease	82,84,88
Picloram	Soybean hypocotyl	^{14}C-ATP, ^{14}C-leucine				Decrease	None	111
Picloram	Corn mesocotyl	^{14}C-orotic acid				Increase		111
IAA	Oat coleoptile	^{32}Pi				None		20
IAA	Pea stem section	^{14}C-glycine	Decrease				Decrease	49

as a relative measure of RNA synthesis indicate that low concentrations of 2,4-D enhance the rate of RNA loss by affecting the rate of RNA breakdown without appreciably affecting apparent RNA synthesis (^{14}C-incorporation). On the other hand, high concentrations of 2,4-D inhibit RNA synthesis, and thus must impair also the mechanism of RNA degradation. In excised elongating soybean hypocotyl, growth-enhancing concentrations of 2,4-D cause the maintenance of RNA and protein at or near the initial level during incubation (85, 88, 173). The enhanced incorporation of ^{14}C-precursor into RNA in response to auxin indicated that auxin maintains the higher RNA content by enhancing synthesis. Growth-inhibiting concentrations of auxin inhibit ^{14}C-precursor incorporation without appreciably affecting the RNA content relative to the control system.

Auxin-enhanced incorporation of radioactive precursors into RNA and protein of many plant tissues has been reported (e.g. 20, 49, 65, 84, 88, 116, 134, 155, 158), but there are some tissues which do not show this response (31, 81). Most auxin-responsive tissues show enhanced synthesis (precursor incorporation) at growth-promoting concentrations and reduced synthesis at growth-inhibitory concentrations of auxin. The net accumulation of RNA in response to auxin leaves little doubt about the capacity of auxin to enhance RNA synthesis (88). The work of Masuda *et al.* (105) indicated that the enhancement of RNA synthesis by auxin is not a result of growth response. Auxin enhanced RNA synthesis in *Avena* coleoptiles in which growth (water uptake) was blocked by isotonic mannitol concentrations.

Reports in the literature on the effect of picloram on RNA and protein synthesis are conflicting. Increases in RNA and protein levels of tissues of susceptible plants such as cucumber and soybean (77, 100) and cotton and cowpea (17) have been found after picloram treatment. Jones (77) and Moreland *et al.* (111), however, were unable to demonstrate picloram effects on RNA and protein synthesis (incorporation of labeled precursors) in corn mesocotyl or soybean hypocotyl tissues.

A precaution in the interpretation of RNA and protein labeling data has been pointed out by Trewavas (159). Long incubations will encourage the growth of contaminating microorganisms in the incubation medium. This problem becomes extremely serious when experiments using isotopic precursors are performed. Hamilton *et al.* (65) reported extensive contamination of coleoptile sections after an incubation period of only 8 hours. The contaminating organisms incorporated high levels of radioactivity into acid-insoluble material. The authors also were able to show that IAA did not affect the rate of incorporation of labeled uridine into contaminating organisms. Key (81) and Key and Shannon (88) have both observed interference by microorganisms in experiments using RNA precursors. Key (81) demonstrated the efficacy of including streptomycin in the incubation medium for reducing bacterial contamination.

d. Requirement of RNA and Protein Biosynthesis for Auxin-Induced Growth

The evidence for a requirement for RNA and protein synthesis during auxin-induced growth has come from studies using inhibitors of

RNA and protein biosynthesis. Since interpretation of most of the results assumes specificity of action of these selected inhibitors, a brief discussion of the action of three of the most frequently used inhibitors is included here.

Probably the most commonly used inhibitor of RNA synthesis is actinomycin D. It is believed to complex specifically with the amino group of deoxyguanosine in DNA, thus inhibiting DNA - dependent RNA synthesis, in cell-free systems from bacteria (26, 58, 129). Actinomycin D has been shown to effectively inhibit RNA synthesis in a wide variety of plants such as *Helianthus* tuber discs (118, 119), oat coleoptiles (65, 105, 116, 119), potato (*Solanum tuberosum* L.) tuber discs (39), *Trillium* microsporocytes (71), and soybean hypocotyls (82, 84). The magnitude and timing of inhibition of RNA synthesis by actinomycin D varies from species to species. It causes as much as 90 to 95 per cent inhibition of RNA synthesis at concentrations of 1 to 10 µg/ml in some plant tissues (82, 88) but less inhibition occurs in other plant tissues (83, 106). Although actinomycin does not inhibit protein synthesis directly, its action does lead to such inhibition (82, 84).

Cycloheximide (actidione) has been shown to effectively inhibit protein synthesis in several organisms including plants (83, 84, 141). The mechanism of inhibition may relate to a direct inhibition of peptide bond formation and to some impairment of the termination or release mechanism (83, 141). Although cycloheximide may be the preferred inhibitor of protein synthesis in plants because of its effectiveness at very low concentration and its rapidity of action (48, 83), some

ribosomal preparations from plants seem to be insensitive (48, 104, 122).

Puromycin inhibits protein synthesis (polypeptide formation) in bacteria by substituting for the amino acid end of amino-acyl tRNA (176); as a result it becomes bound to the nascent protein (113). Since protein synthesis in plants involves tRNA also (23), the inhibitory mechanism is doubtless the same. Puromycin has been shown to inhibit protein synthesis in a variety of plant tissues (82, 118, 119), but a high concentration (10^{-4} to 10^{-3} M) generally is required for effective inhibition (82, 118). Because of the high concentrations required to accomplish inhibition of protein synthesis, puromycin should be used with extreme caution as a specific inhibitor of protein synthesis.

A growing body of evidence shows that continued synthesis of RNA and protein is essential for continued growth of intact and excised plant tissues. Key *et al.* (84) and Nooden and Thimann (118, 119) have shown that normal (control) and auxin-induced growth of soybean hypocotyl and *Avena* coleoptile are inhibited by actinomycin D. Similar observations have now been made in many excised plant tissues (38, 43, 106, 115, 118, 119, 124). It has been demonstrated that continued RNA synthesis is essential for continued growth of excised plant tissues (43, 82, 84, 116) and that RNA synthesis can be inhibited to a considerable extent without affecting this growth (84, 86, 119). Actinomycin at low concentrations, up to about 0.2 µg/ml, inhibited RNA synthesis by 30 to 40 per cent over 8 hours without affecting cell elongation in soybean hypocotyls (86). Actinomycin D at low concentrations (e.g. 1 µg/ml or less) selectively inhibits rRNA synthesis (83). Higher concentrations inhibited RNA synthesis and cell elongation in parallel

fashion. These studies with actinomycin D, and others with 5-fluro-uracil (which selectively inhibits synthesis of rRNA and sRNA (84, 86) on excised soybean hypocotyl (84, 86) and artichoke tuber (118) tissue, have indicated that the requirement for RNA synthesis to support growth is restricted to the synthesis of D-RNA, properties of which suggest that it is identical, at least in part, to mRNA (86). Viewed as a whole, the available evidence implies that mRNA synthesis is required for continued growth of excised plant tissues.

The requirement for RNA synthesis during growth implies a requirement for protein synthesis (83). Indeed a wide range of protein synthesis inhibitors (chloramphenicol, cycloheximide, and puromycin) inhibit cell elongation of both excised and intact plant tissues (43, 82, 84, 115, 118, 119, 124). There is a close parallel between the inhibition of growth and protein synthesis by cycloheximide in soybean hypocotyl (84). Results on inhibition of protein synthesis and growth in different plant tissues by puromycin are conflicting, with a strong inhibition in soybean hypocotyl (82) and oat coleoptile (119), and little inhibition in pea stem sections (43, 119, 124).

The foregoing discussion dealt with the general requirement for the continued synthesis of RNA and protein for continued growth. The available data do not allow for a definitive conclusion regarding the relationship of this requirement to the regulation of growth by auxin, but several observations (84, 105, 118, 119) indicate that the ability of auxin to enhance the rate of cell enlargement is dependent upon new RNA and protein synthesis and that auxin can not cause a growth response by utilizing pre-existing RNA or protein. The auxin-induced

growth is inhibited much more than the endogenous growth by inhibitors of RNA and protein synthesis. For example, in soybean hypocotyl, corn mesocotyl and corn coleoptile, actinomycin at 5 to 10 µg/ml caused a 60 to 70 per cent inhibition in auxin-induced growth but during the same interval endogenous growth was inhibited by only 5 to 10 per cent (82, 83, 84, 86). Cycloheximide treatment of soybean hypocotyl caused similar responses (84). If these tissues were pretreated with auxin to obtain the typical two- or three-fold increase in growth rate prior to the addition of actinomycin or cycloheximide, the auxin-induced component of growth was maintained to about the same extent for the same time as the original normal growth (84). Since the ability of auxin to enhance growth is directly proportional to the level of D-RNA synthesis (84), this has been interpreted (83) to mean that auxin-treated tissues contain about three times as much "growth essential" RNA (and/or protein) as control tissues. This, of course, assumes that auxin does not affect the stability of the "growth essential" RNA (and protein).

There is not much information on specific effects of auxin on protein synthesis. Effects on protein synthesis in excised tissues (measured by ^{14}C-amino acid incorporation) range from small increases to no effect. Fan and Maclachlan (51) showed a very large specific enhancement of cellulase activity in pea in response to IAA, an effect which was blocked when RNA synthesis or protein synthesis was inhibited by actinomycin D. Low levels of 2,4-D caused a small enhancement of ^{14}C-leucine incorporation into soybean hypocotyl (82) and *Avena* coleoptile (119) protein. This effect on protein synthesis (and growth

in response to auxin) was blocked by actinomycin D, while protein synthesis in control tissue was inhibited only slightly during the same interval.

C. Uptake, Translocation, and Metabolism of Herbicides

I. Uptake by Excised Plant Tissues

With the object of elucidating the processes by which plant growth regulators exert control over plant growth and metabolism, the pattern of uptake has interested a number of workers (37, 75, 164, 165, 166). Excised plant segments can take up growth regulators from a liquid medium and accumulate it in the tissues to a concentration higher than that of the external solution (75). From comparisons of the rates of accumulation of chlorinated benzoic acids by plant segments of different length, Venis and Blackman (164) concluded that uptake proceeds largely or wholly via the cut surfaces. A similar conclusion was drawn by Vanden Born (161) from studies on entry of dicamba into oat and barley seedlings via intact coleoptiles and from cut surfaces.

Christie and Leopold (37) and Johnson and Bonner (75) suggested that the course of uptake of the growth regulators IAA and 2,4-D by plant tissues consists of at least two separable processes, (a) an initial rapid uptake probably by diffusion into the free space of the tissue, and (b) a continued accumulation process which involves a metabolic component. The involvement of metabolic activities has been repeatedly shown using metabolic inhibitors, such as DNP and KCN (37, 74, 75, 161). The rate of continuing uptake is reduced by metabolic

inhibitors while the initial diffusion generally is inhibited only slightly by these same inhibitors, if at all. Temperature markedly affects the rate of uptake of growth regulators (37, 75, 161). Vanden Born (161) reported that uptake and release of dicamba by oat and barley coleoptiles at 10°C were 50 to 60 per cent of the corresponding values at 25°C.

From extensive studies on the uptake of growth regulators by excised plant segments, Blackman and his colleagues (74, 138, 164, 165, 166) concluded that the pattern of uptake of a growth regulator is dependent on plant species and, for a particular species, is related to chemical structure which in turn determines physiological activity. For example, the rate of uptake of phenoxyacetic acid and benzoic acid compounds that have strong auxin properties, such as 2,4-D, 2,4,5-T, and TCBA (2,3,6-trichlorobenzoic acid), is high initially but subsequently falls markedly, and for some species it may become negative so that a net loss of the growth regulator to the medium ensues. This was referred to as 'Type 1 accumulation' (138) and is considered an unstable mechanism of accumulation (165). Venis and Blackman (165) presented evidence that the quaternary ammonium groups associated with the phosphatide components of cellular membranes provide cationic sites suitable for eletrostatic interaction with the anion of the growth regulator. Venis and Blackman (165) further suggested that the unstable nature of this accumulation mechanism is due to enzymatic degradation of phosphatidylcholine by phospholipase D. Such degradation destroys the effectiveness of the choline quaternary ammonium groups as a binding site.

Very little work has been reported on the uptake of picloram by excised plant tissues. Baur and Bovey (16) and Swanson and Baur (154) suggested that the uptake of picloram by potato tuber discs occurs in part via an unstable accumulation mechanism similar to that proposed by Venis and Blackman (165, 166) for other auxinic herbicides. Although inhibitor studies are needed to characterize further the nature of picloram uptake, from the experiments of Swanson and Baur (154) it appears that there is both a strong physical diffusion component and an associated metabolic component to uptake.

II. Herbicide Translocation in Plants

After many years of work with radioactive herbicides, Crafts and Yamaguchi (42) concluded that systemic distribution of foliage-applied phloem-mobile compounds follows the way of assimilate translocation. The consistent bypassing of mature leaves, the high concentration in young growing shoot tips, root tips, and intercalary meristems, and the reversibility of flow brought about by proper manipulation, are interpreted as indication of a mass-flow type of mechanism (42).

Herbicides can be divided into several categories on the basis of translocation characteristics (88): (a) those that move slightly if at all, e.g. oil and oil-soluble esters of 2,4-D and 2.4,5-T, diquat; (b) those that are translocated in the symplast primarily, e.g. the acid and salt forms of 2,4-D and 2,4,5-T; (c) those that are translocated in the apoplast only, e.g. fenuron, and other substituted ureas and symmetrical triazines; (d) those that move in both the symplast

and apoplast, e.g. amitrole, maleic hydrazide. Symplastic movement is of greatest importance following leaf application, while apoplastic movement is of greatest importance following root application. Compounds possessing the ability to move in both the symplast and the apoplast should have great potential as herbicides.

Limited available information suggests that picloram is translocated readily in plants. Picloram was originally characterized as comparable to 2,4-D and 2,4,5-T in absorption and translocation by plants (63). However, later studies in field bindweed (2,3) and bean (131) showed that movement of picloram is greater than that of 2,4-D. Bovey *et al.* (24) reported that most of the picloram applied to foliage of huisache(*Acacia farnesiana* (L) Willd.) was found in and on the leaves 30 days after treatment, and that a period of 24 hours was required to move lethal amounts of picloram into stem and root tissues. A similar period of root exposure to picloram solution was required to kill 20-day old seedlings of huisache and honey mesquite (*Prosopis juliflora* L.) (15). Reid and Hurtt (131) observed that translocation of ^{14}C-picloram from roots to apical parts of beans was very rapid and that the herbicide was accumulated preferentially in the terminal bud and first trifoliolate leaflets. Very little picloram was transported to the primary leaves and only at longer treatment periods. Hurtt and Foy (72) provided evidence that picloram was foliarly-absorbed, phloem-translocated, and excreted from the roots to the surrounding medium by picloram-treated bean plants.

Canada thistle foliage and roots readily absorbed picloram,

after which it was translocated by both phloem and xylem (146). Picloram tended to accumulate in young, growing leaves following both foliar and root uptake. After foliage application, small amounts of picloram were exuded by the roots into the surrounding soil, and it also moved readily through connecting creeping roots from one shoot to another.

III. Herbicide Metabolism in Plants

It is known that plants have the ability to detoxify certain exogenously applied compounds through metabolic activity (78). It now has been demonstrated that decomposition of herbicides by metabolic processes is of importance in the selective action of a number of these chemicals and in determining the amount of residue that may be left on crops harvested for human utilization (33, 57, 78).

The fate of herbicides in plants has been reviewed by a number of authors in recent years (25, 57, 78, 153). In general, four major pathways are involved in herbicidal metabolism, i.e. conjugation, oxidation, reduction, and hydrolysis. As indicated by Freed and Montgomery (57), "While a given metabolic path may predominate for a particular compound, it is quite common for any one or more of the detoxification mechanisms to be simultaneously operative. Thus, while a compound may undergo oxidative metabolism principally, it may be found that a portion of the administered drug will simultaneously be undergoing conjugation, reduction or hydrolysis".

In most instances, the metabolism of herbicides in plants is considered to be an inactivation or detoxification process. There

are some chemicals, however, which are initially inactive but are rendered phytotoxic by the metabolic action of the treated plant. The beta-oxidation of 4-(2,4-dichlorophenoxy)butyric acid to 2,4-D is a classical example. Another example is the production in the plant of toxic free radicals from the bipyridilium quaternary salts known as diquat and paraquat (25).

Compared to other herbicides such as the phenoxy acids, the triazines, the substituted carbamates, and the benzoic acid derivatives, very little information is available on the fate of picloram in plants. Meikle *et al.* (107) reported that the major portion of picloram applied to cotton plants could be extracted unchanged. Only 3 per cent of the total extractable radioactivity was associated with insoluble protein. This radioactivity was liberated from the protein on acid hydrolysis and was identified as unaltered picloram. The rate of $^{14}CO_2$ evolution from ^{14}C-picloram by cotton plants over a period of 15 days was negligible. Redmann *et al.* (128) found small amounts of oxalic acid, 4-amino-3,5-dichloro-6-hydroxypicolinic acid, and 4-amino-2,3,5-trichloropyridine as metabolites of picloram in wheat plants 90 days after treatment with the herbicide. In short term experiments, lasting up to 24 hours, only unaltered picloram was obtained from ^{14}C-picloram-treated pea roots (143) and bean petioles and pea stem sections (70).

MATERIALS AND METHODS

A. Materials

I. Chemicals

The herbicide used in the experiments described was a commercial formulation of the potassium salt of picloram (4-amino-3,5,6-trichloropicolinic acid) (Tordon 22 K*). The radioactive picloram (^{14}C-picloram) used was labeled with carbon-14 in the carboxyl group with a specific activity of 1.03 mCi/m mole and was obtained as a gift from Dow Chemical Co., Inc. The potassium salt of ^{14}C-picloram was prepared by mixing together appropriate quantities of labeled picloram and Tordon 22K blank formulation (supplied with ^{14}C-picloram). $Ba^{14}CO_3$ (specific activity: 50.2 mCi/m mole) was obtained from the Radiochemical Center. Adenosine-8-^{14}C-triphosphosphate, tetrasodium salt (^{14}C-8-ATP, specific activity: 41.9 mCi/m mole) and ^{14}C-U-L-leucine (specific activity: 260 mCi/m mole), and hyamine hydroxide were obtained from New England Nuclear Corporation. Other chemicals were generally of the highest purity available and from the following sources: actinomycin D, adenosine-5'-triphosphate (disodium salt), bovine serum albumin, L-leucine, puromycin dihydrochloride, gibberellic acid (K-salt), ribonucleic acid (reagent grade) and orcinol (3,5-dihydroxytoluene)

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* Trade mark of Dow Chemical Co., Inc.

from Nutritional Biochemicals Corporation; cycloheximide from Aldrich Chemical Co., Inc., streptomycin sulfate from Merck and Sharp Co. All other chemicals were obtained as reagent grade from Fisher Scientific Co. Ltd. All water used for preparing solutions was double distilled deionized unless specified otherwise.

II. Plant Material

Plants used were Canada thistle (*Cirsium arvense* (L.) Scop.), soybean (*Glycine max* (L.) Merr. cultivar Harosoy 63), barley (*Hordeum vulgare* L. cultivar Parkland), and corn (*Zea mays* L., cultivar Morden 77). Soybean seed was obtained as a gift from Harrow Research Station, Harrow, Ontario. Corn seed was purchased from Robertson Seeds Ltd., Edmonton.

Canada thistle plants were vegetatively propagated from a single plant. Individual plants were grown from creeping root sections about 8 cm long. The plants were grown in 15-cm plastic pots containing a 3:2:1 mixture of Malmo clay loam, sand, and peat, in a growth chamber at 23°C, with 16-hour light and 50-60% relative humidity unless specified otherwise. Light intensity at plant level was 10760 lux from a mixture of fluorescent and incandescent lamps. Soybean, barley, and corn plants were grown from seed which was previously dusted with the fungicide Orthocide* (N-trichloromethylthiotetrahydrophthalimide). Growing conditions were the same as those described for Canada thistle, except

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* Trade mark of Chevron Chemical Co.

that the light intensity at plant level was 17220 lux. For $^{14}CO_2$-fixation and photosynthate translocation studies only, soybean and corn plants were grown in a 1:1 mixture of sand and vermiculite supplemented with a complete fertilizer.

For isolated tissue studies soybean and barley seeds were soaked in water for 2-3 hours and then germinated in the dark at 25°C in a germination cabinet. Soybean and barley seeds were planted in vermiculite and between layers of paper towels, respectively, in 35.5 x 23 cm plastic trays and irrigated with 10^{-3} M $CaCl_2$ solution. After 60 hr the paper towels were removed from the barley seeds and the seedlings were allowed to grow for another 24 hr.

B. Comparison of Plant Susceptibility to Picloram

The sensitivity to picloram of Canada thistle, soybean, and barley was determined by foliar application of the potassium salt of the herbicide. Greenhouse-grown (in June-July with approximately 16 hr sunlight at 75°F) 5-week-old Canada thistle and 12-day-old soybean and barley plants were sprayed with different concentrations of picloram with a cabinet sprayer. The rates of application varied from 0.063 to 8 oz/A (ounces per acre) for soybean, 0.25 to 32 oz/A for Canada thistle, and 1 to 64 oz/A for barley. All the spray treatments were applied in 10 gal/A of water. Subsequently, the plants were kept in the greenhouse for observation. Two weeks after treatment the above-ground parts of the plants were harvested, oven dried, and weighed.

C. $^{14}CO_2$-Fixation Studies

I. Light-Fixation

Uniform 5-week-old Canada thistle and 10-day-old soybean and corn plants were transferred to one-half strength Eliasson nutrient solution (47). After a 2-day acclimatization period, two plants of each species were transferred to 100 ml of nutrient solution containing 1, 10, or 20 µg/ml of the potassium salt of picloram. Two other plants were used as controls and were transferred to 100 ml of fresh nutrient solution without herbicide. All plants were kept in the light for 24 hr.

For $^{14}CO_2$-fixation two full-grown leaves per treatment were selected at random, outlined on paper to measure leaf area, excised under water, and placed in 25-ml beakers with deionized water. The beakers were transferred to a specially constructed photosynthesis chamber, a 4-litre glass container sealed with a glass cover, and conditioned for 10 minutes under reduced light. $^{14}CO_2$ was generated in the chamber by the slow addition of 10 per cent aqueous lactic acid from a hypodermic syringe to 3.90 mg $Ba^{14}CO_3$. The leaves were allowed to fix carbon for 30 minutes at 23 ± 0.5°C under 10220 lux of fluorescent light at leaf level. At the end of 30 minutes each leaf was removed from the photosynthesis chamber and dropped into 100 ml boiling 80 per cent ethanol.

II. Dark-Fixation

For dark-fixation of $^{14}CO_2$ the nutrient solutions containing picloram were discarded and replaced with fresh nutrient solution after 24 hr in light. Two full-grown leaves were chosen at random, marked, and outlined on paper to measure leaf area. After these preparations the plants were placed in darkness for 24 hr. The marked leaves then were excised under a dim red light, exposed to $^{14}CO_2$ for 1 hr in total darkness at 20°C, in the manner described above, and killed in boiling 80 per cent ethanol.

III. Extraction and Sample Preparation

The leaves obtained after $^{14}CO_2$-fixation were homogenized in 80 per cent ethanol in a Waring blender. The homogenate was filtered quantitatively through Whatman No. 1 filter paper in a Buchner funnel and made to a final volume of 250 ml with 80 per cent ethanol. Aliquots of 0.5 ml were used for radioactivity determinations. The amount of carbon fixed per experimental period per square centimeter of leaf surface was then calculated from the radioactivity measurements.

IV. Chromatographic Separation of ^{14}C-Assimilates

The distribution of radioactivity among different compounds after $^{14}CO_2$-fixation was determined by the method of Bassham and Calvin (14). The ethanol extracts were concentrated under reduced pressure at about 40°C. The resulting material was dissolved in deionized water and extracted three times with petroleum ether. The aqueous layer

was concentrated under vacuum and the contents were made to 2 ml in 2.5×10^{-5} M K_2HPO_4 - KH_2PO_4 solution (this small quantity of added phosphate improves the subsequent chromatography (14)).

Sheets of Whatman No. 3 filter paper, 40 x 50 cm, were used for paper chromatography. A small amount of the extract, containing a known amount of radioactivity, was applied to an area of 4 x 10 mm at a distance of 6 cm from two edges of the paper and with the long axis of the area of application (origin) parallel to the short axis of the paper. Drying of the extract was facilitated by a stream of warm air. The chromatograms were placed in the chromatography tank for four hours' saturation and then were developed two-dimensionally in a descending manner. The solvent used in the first dimension was made up of 72 per cent liquid phenol (90 per cent) and 28 per cent water (v/v). After drying in air the chromatogram was developed in the second dimension using a solvent system made up by mixing equal parts of a mixture of n-butanol (1246 ml) + water (84 ml) and a solution of propionic acid (620 ml) + water (790 ml). The developed paper chromatograms were air-dried and exposed to Ilford No-Screen X-ray film for two weeks. Films were developed in Ansco Liquadol developer for four minutes and fixed for ten minutes or longer in Kodak fixer. Spots containing labeled compounds were cut from the chromatograms and left overnight in scintillation vials with scintillation fluid before counting. Their relative activities then were determined in a liquid scintillation counter.

V. Identification of Radioactive Compounds

Radioactive compounds were identified by conventional chromatographic techniques (14, 22). Spots containing labeled compounds were eluted from the paper chromatograms and chromatographed separately on filter paper strips (4 cm wide) using phenol:water and butanol: propionic acid:water as solvents (concentrations as before, section C-IV). Reference compounds were chromatographed at the same time. Visualization of the non-radioactive reference compounds on the chromatograms was done with color developing agents (22) as follows:

Amino acids: 0.3 per cent ninhydrin in 95 per cent ethanol.

Organic acids: 1 g aniline and 1 g xylose dissolved in a minimal amount of water and made to 100 ml with methanol. After spraying the chromatograms were dried at room temperature and heated to 105°C for 30 minutes.

Sugars: 0.25 g benzidine, 5 ml glacial acetic acid, and 5 ml trichloroacetic acid made to 50 ml with 95 per cent ethanol. After spraying chromatograms were heated to 100°C.

D. Liquid Scintillation Counting

All the quantitative determinations of radioactivity were done in a Nuclear Chicago Mark I liquid scintillation spectrometer. The scintillation fluid used was prepared by dissolving 120 g naphthalene, 6 g 2,5-diphenyloxazol (PPO), and 0.5 g 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP) in 1,4-dioxane to make up one litre. The samples usually

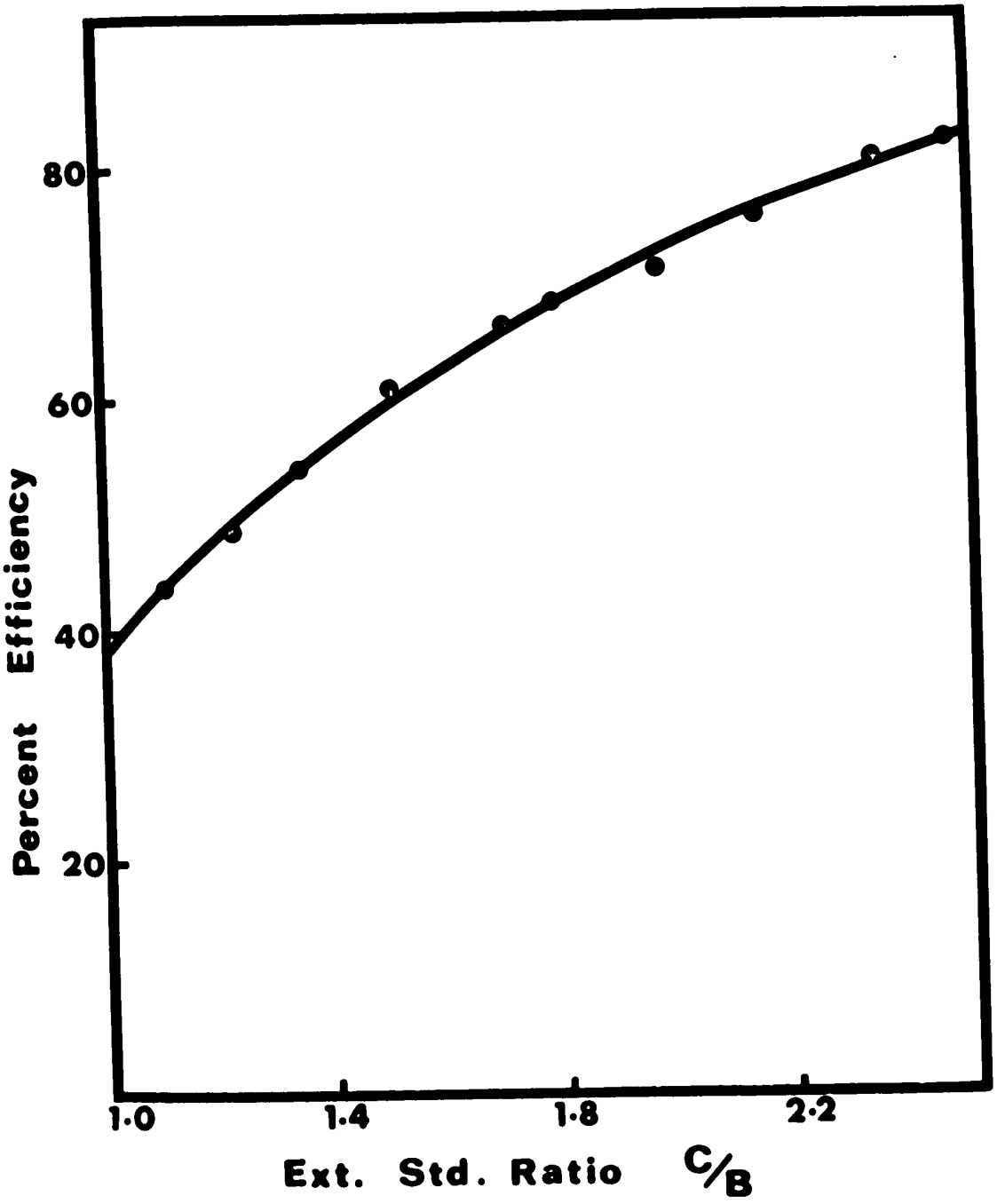
were counted in 15 ml of this solution at 8°C.

Counting efficiency was determined by the external standard method (168). Standard quenching curves were prepared by counting a series of differently quenched samples, containing a known amount of ^{14}C, in two channels set to monitor different portions of the energy spectrum. The channels ratio of the external standard counts then was plotted against the observed counting efficiency to get the quenching curve. One such curve is shown in Figure 1. The quenching agents used in the standard cuve preparation were extracts of untreated plants, tissue homogenate, ethanol or water, depending on the nature of the samples to be counted. From the appropriate standard curve, the counting efficiency for each sample was determined and the observed radioactivity in cpm then was converted to dpm.

E. Translocation of ^{14}C-Assimilates

For studies on translocation of labeled photosynthate, Canada thistle, soybean, and corn plants were transferred to 100 ml nutrient solution containing 0, 1, or 10 µg/ml picloram. After 24 hr they were exposed, three plants at a time (one for each concentration), to 0.5 mCi of $^{14}CO_2$ in a polyethylene bag under the conditions described earlier for light-fixation studies (section C-1). Exposure time was 10 minutes. Duplicate plants were used for each treatment. After $^{14}CO_2$ exposure, plants were transferred to fresh nutrient solution without picloram and returned to the growth chamber for 0, 4, or 24 hr to permit translocation of the labeled photosynthate. Distribution of

Figure 1. One of the quenching curves used for carbon-14 assay in Nuclear Chicago Mark I liquid scintillation spectrometer. Samples quenched with ethanol extracts of untreated Canada thistle plants. Efficiencies determined using ^{14}C-$NaHCO_3$ standard. Counting time 20 minutes; temperature 8°C. Discriminator settings: L-U, L = 0.0; U = 9.9. Attenuation: channel B: A 640; channel C: D 912.


80
60
40
20
Percent Efficiency
1.0
1.4
1.8
2.2
Ext. Std. Ratio C/B

radioactivity in the plants was determined by autoradiography as described by Crafts and Yamaguchi (42). After harvesting, plants were freeze-killed with crushed dry ice, and freeze-dried. The drying process was completed in about two weeks. The dried plant materials were humidified, mounted and flattened, and then autoradiographed using Ilford or Kodak No-Screen X-ray films. Exposure time was one week. To check possible artifacts, untreated plants were exposed to X-ray films in a similar way.

F. Chlorophyll, RNA, and Protein Content of Intact Plants

I. Treatment of Plants

Pots with uniform 5-week-old Canada thistle (1 plant/pot) and 10-day-old soybean (6 plants/pot) and barley (15 plants/pot) were sprayed with 2 ml 0.01 M phosphate buffer (pH 6.0) containing 250 μg/ml picloram and 0.5 per cent Atlox 210* (a nonionic blended surfactant) with a small hand atomizer. Similar plants sprayed with phosphate buffer and Atlox 210, without picloram, were used as controls. Two replications were used for each treatment and two pots made up one replication. The leaves including the apex (in soybean and Canada thistle) were harvested 1 or 3 days after spraying and were washed thoroughly in deionized water. The plant material was freeze-dried, ground in a Wiley mill to pass through a 60 mesh screen, and stored in a desiccator at -20° C until analyzed.

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* Trade mark of Atlas Chemical Industries, Inc.

II. Extraction and Sample Preparation

a. Chlorophyll

Approximately 0.5 g dry plant material was ground in 80 per cent acetone with fine sand as an abrasive using a mortar and pestle. The suspension was transferred to a Buchner funnel and filtered through a double layer of Whatman No. 1 filter paper. The samples were made to an appropriate volume and an aliquot was used immediately to measure the chlorophyll concentration.

b. RNA and Protein

For extraction of RNA and protein approximately 1 g dry plant material was homogenized with ice-cold 0.1 M Tris-HCl buffer (pH 7.4) in a glass homogenizer with a power driven teflon pestle. The slurry was squeezed through 6 layers of cheese cloth. The cleared homogenate was divided into two equal portions and transferred to 50 ml polyethylene centrifuge tubes. RNA from one aliquot of the homogenate was isolated by a modified Smillie and Krotkov (150) method as described by Key and Shannon (88). The homogenate was mixed with equal volumes of cold 0.6 N perchloric acid ($HClO_4$). After standing at 2-4° C for 1 hr or more, the precipitate was sedimented by centrifuging at 15000 x g for 15 minutes in a Sorvall SS-3 centrifuge. All above steps including centrifugation were carried out at 2 to 4° C. The resulting pellet was washed in 5 ml of 0.2 N perchloric acid and recentrifuged. The washed pellet was extracted twice with 5 ml portions of 2:2:1 ethanol:ether: chloroform at 37° C for 30 minutes to remove lipids. RNA in the pellet

was hydrolyzed in 0.3 N KOH at 37° C for 18 hr. After chilling, perchloric acid was added to a final concentration of about 0.3 N followed by centrifugation to remove the $KClO_4$ precipitate, protein, and DNA. The samples were then made to an appropriate volume with Tris-HCl buffer and an aliquot was used to measure the RNA concentration.

Protein in the other aliquot of the homogenate was precipitated with ice cold trichloroacetic acid (TCA) with a final concentration of 10 per cent. The precipitate was collected by centrifugation as described above for RNA. The resulting pellet was washed once with 5 per cent TCA by resuspension and resedimentation, and decolorized and defatted by extracting three times with acetone. The precipitate was then dissolved in a small volume of 1 N NaOH, made to an appropriate volume with Tris-HCl buffer and an aliquot was used to measure the protein concentration.

III. Measurement

All optical density measurements were carried out in a Bausch and Lomb Spectronic 20 spectrophotometer.

a. Chlorophyll

The optical density of the extracts was measured at 652 mμ. The chlorophyll concentration was calculated by using the following equation given by Bruinsma (27).

$$Ca + b = \frac{1000\ A_{652}}{36.0} = 27.8\ A_{652}$$

where C_{a+b} = Total chlorophyll (a+b) concentration in mg/ml.

A_{652} = Absorbance or optical density at 652 mμ.

b. RNA

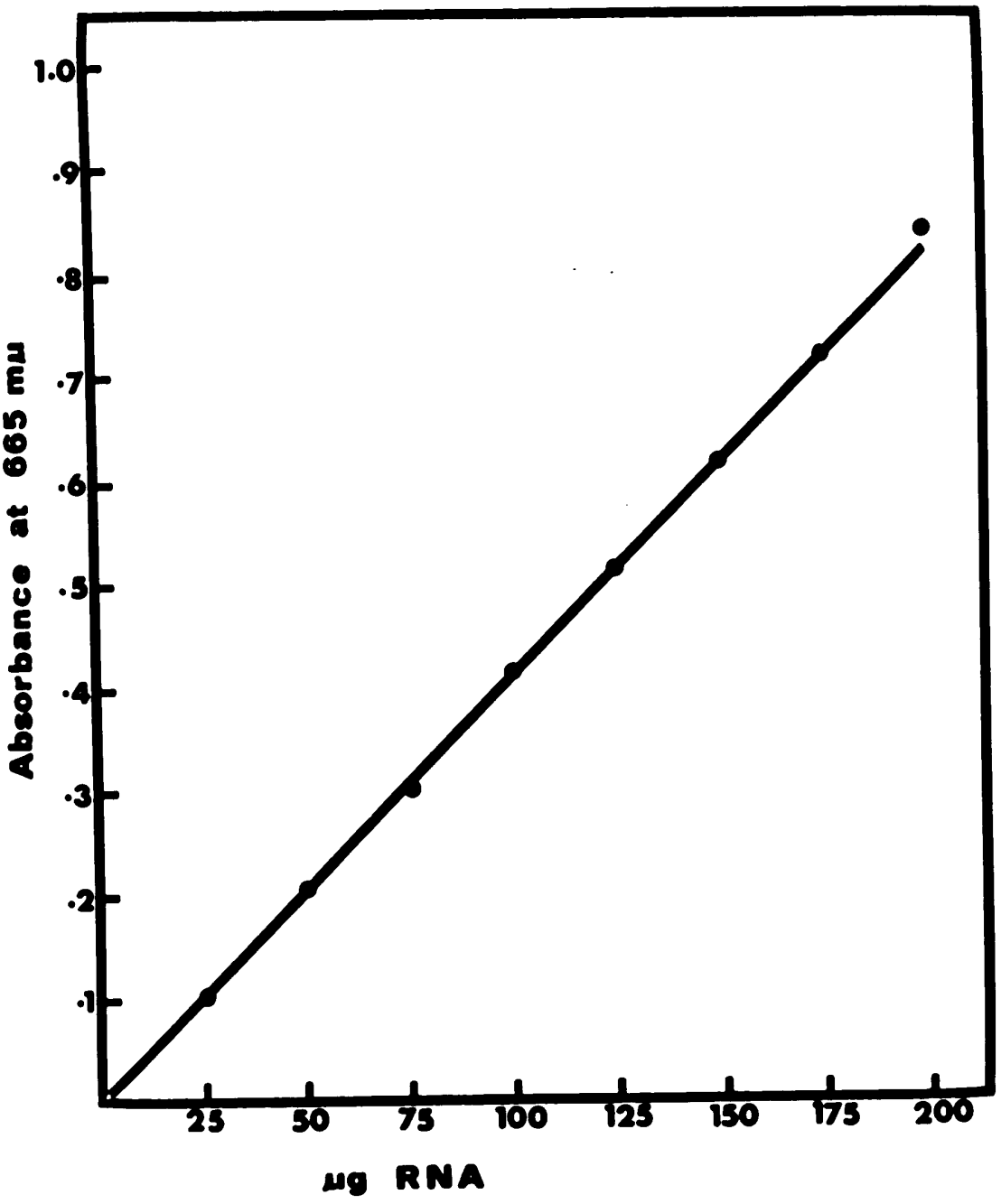
RNA in the samples was determined by the colorimetric procedure of Dische (44). To 1.5 ml of sample solution 3.0 ml of orcinol reagent was added. The reagent was prepared by dissolving 100 mg of $FeCl_3 \cdot 6H_2O$ in 100 ml of conc. HCl (sp. gr. 1.19), and adding 3.5 ml of a 6 per cent solution of orcinol in ethanol. The reaction mixture, in test tubes, was heated in a boiling water bath for 30 minutes, and then cooled in running tap water. The optical densities of the solutions at 665 mμ were determined against a blank consisting of 0.1 M Tris-HCl buffer (pH 7.4).

A standard curve was prepared from a series of known concentrations of RNA by using the above procedure. Each point on the standard curve (Figure 2) is an average of three determinations.

c. Protein

Protein concentration was determined by the method of Lowry *et al.* (99). To test tubes containing a 1.0 ml aliquot of the sample solution was added 5 ml of a mixture of 1.0 ml of 0.5 per cent $CuSO_4 \cdot 5H_2O$ in 1 per cent potassium tartrate + 50 ml of 2 per cent Na_2CO_3 in 0.1 N NaOH, and after 10 minutes 0.5 ml of aqueous Folin-phenol (1:1, v/v) was added. The intensity of the blue color, developed after an incubation of 30 minutes at room temperature, was measured at 630

Figure 2. Standard curve for RNA determination. RNA was estimated by the procedure of Dische (44). RNA concentration is expressed as µg per ml.


1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
Absorbance at 665 mμ
25
50
75
100
125
150
175
200
μg RNA

mμ. Quantitative estimation of protein was obtained from a standard curve (Figure 3) prepared in a similar manner by using bovine serum albumin.

G. RNA and Protein Biosynthesis by Excised Plant Tissues

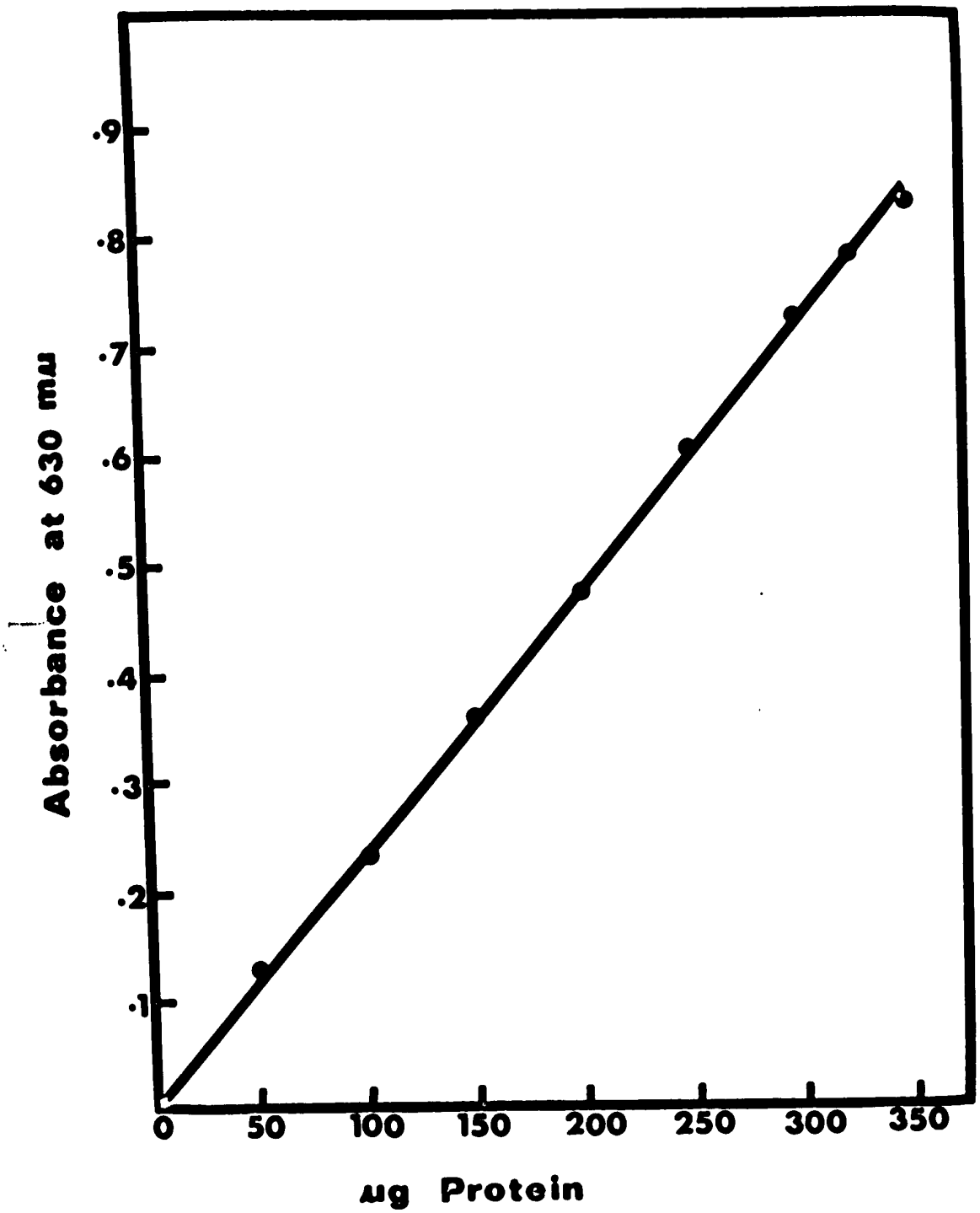
I. Tissue Preparation

Soybean hypocotyl and barley coleoptile sections, 13 mm in length, were excised from the elongating zone (5 mm below the hook of soybean hypocotyl and the tip of barley coleoptile) of 3 1/2-day-old dark grown seedlings. Barley leaf sections (13 mm in length) were excised from 10-day-old seedlings grown in growth chamber. Leaf discs, 14 mm in diameter, were punched with a cork borer from fully expanded leaves of 5-week-old Canada thistle plants. All excised sections were maintained in ice-cold distilled water for 1/2 hr before incubation.

II. Incubation

Twelve soybean hypocotyl or thirty barley coleoptile sections, with a total weight of approximately 0.5 g, were incubated in 5 ml solution in 50 ml Erlenmeyer flasks on a water bath shaker in total darkness at 25°C. Sterilized buffer solutions and glassware were used in all incubation experiments. The incubation medium consisted of 0.01 M phosphate buffer (pH 6.0) with 1 per cent sucrose and 20 μg/ml streptomycin sulfate. Picloram concentration was 0, 10, 100, or 500 μg/ml. ^{14}C-U-leucine at 0.05 μCi/ml and ^{14}C-8-ATP at 0.1 μCi/ml were included in the incubation medium.

Figure 3. Standard curve for protein determination. Protein was estimated by the procedure of Lowry *et al.* (99) using bovine serum albumin. Protein concentration is expressed as µg per ml.


Absorbance at 630 mμ
.9
.8
.7
.6
.5
.4
.3
.2
.1
0
50
100
150
200
250
300
350
μg Protein

Incorporation of a labeled nucleotide into RNA and of a labeled amino acid into a polypeptide chain has been widely used to measure and study *de novo* RNA and protein synthesis in a variety of plant tissues. Many investigators (82, 84, 102, 111) have used ^{14}C-ATP and ^{14}C-leucine extensively for RNA and protein synthesis studies.

In excised tissue studies, streptomycin sulfate (20 µg/ml) was used to control bacterial growth. Streptomycin at a concentration (20 µg/ml) used in these experiments has been reported to control bacterial growth without seriously affecting precursor incorporation into RNA and protein of higher plant tissues (65, 81, 82, 133).

Fifteen Canada thistle discs or thirty barley leaf sections were incubated in 30 ml of the above incubation medium in petri dishes at 23° ± 1° C under 10220 lux of fluorescent light.

Three replications for each treatment were used in all excised tissue studies. After the desired periods of incubation, ranging from 1 to 24 hr, the sections were thoroughly washed for three minutes in running tap water and then for 1 minute in distilled water. Sections were blotted dry, weighed, and immediately frozen at -20°C.

III. Extraction and Sample Preparation

Frozen tissue was ground at 2-4° C in a glass homogenizer with a power-driven teflon pestle. The grinding medium consisted of 0.1 M Tris-HCl buffer (pH 7.4) with 0.2 mg/ml unlabeled ATP or L-leucine. RNA or protein in the filtered homogenate was extracted as outlined in

section F-II and the concentration and/or radioactivity was determined. The supernatant remaining after RNA or protein extraction was saved for radioactivity determination. When the tissue, following incubation, was to be separated into particulate and soluble fractions, sections were ground in a pre-chilled mortar and pestle in the above grinding medium which, in addition, contained 0.33 M sucrose and 0.02 M $MgCl_2$. Particulate and soluble fractions were separated by centrifuging at 20000 x g for 30 minutes. The pellet thus obtained was washed twice with small volumes of the grinding medium, recentrifuged each time and the washings added to the soluble fraction. RNA and protein from each fraction were then extracted and their radioactivity was determined.

IV. Radioactivity Measurement

Radioactivity in 0.5 ml aliquots of RNA, protein, and supernatant fractions was measured by liquid scintillation counting (section D). Uptake of ^{14}C-8-ATP and ^{14}C-leucine and their incorporation into RNA and protein, respectively, were calculated as dpm/g fresh weight of tissue.

H. Uptake and Subcellular Distribution of ^{14}C-Picloram by Excised Plant Tissues

I. Uptake

Soybean hypocotyls, barley coleoptiles, barley leaf sections, and Canada thistle leaf discs were prepared and incubated in a manner similar to that described in section G-I and G-II. Incubation was

carried out in 0.01 M phosphate buffer (pH 6.0) containing 10 µg/ml streptomycin sulfate and 0.05 µCi/ml picloram-^{14}C. After the desired periods of incubation, the sections were washed thoroughly in distilled water, blotted dry, and immediately homogenized in acetone in a glass homogenizer with a power-driven teflon pestle. The suspension was quantitatively transferred to scintillation vials and the excess acetone evaporated in a warm air stream from a hair drier. The radioactivity of each sample was determined in a liquid scintillation counter (section D).

II. Subcellular Distribution

Plant tissue sections were incubated with ^{14}C-picloram as described above for uptake. After 8 hr of incubation the sections were washed in distilled water, blotted dry, and frozen at -20°C. The frozen material was ground with ice cold 0.1 M Tris-HCl buffer (pH 7.4) containing 0.33 M sucrose +0.02 M $MgCl_2$, in a pre-chilled mortar and pestle. The slurry was squeezed through six layers of cheese cloth and subjected to differential centrifugation as follows.

250 x g for 5 min. - Nuclei-rich (leaf tissue).

4500 x g for 10 min. - Chloroplast-rich (leaf tissue) or nuclei-rich (hypocotyl or coleoptile).

20000 x g for 30 min. - mitochondria-rich.

supernatant - soluble fraction.

Each time after centrifugation the pellet was washed twice in a small

volume of grinding medium, and recentrifuged, and the washings were added to the subsequent fraction. Each fraction was made to an appropriate volume and 0.5 ml aliquots were used for radioactivity determination by liquid scintillation counting.

I. Translocation of ^{14}C-Picloram in Plants

I. Autoradiography

Autoradiographic studies of picloram translocation in plants following foliar or root application were carried out by the method described by Crafts and Yamaguchi (42).

Uniform 5-week-old Canada thistle and 12-day-old soybean and barley plants were treated with the herbicide by placing 10 µl of ^{14}C-picloram solution in 50 per cent ethanol on the upper surface of a full-grown leaf. Each droplet was applied in a small lanolin ring on the midrib in the center of each leaf. The dose of picloram was 0.1 µCi (approximately 23.5 µg). The treatment was applied in the greenhouse and treated plants were kept there until they were harvested, 2 hr to 4 days later. At the end of the treatment periods the treated spots were covered with a piece of masking tape to prevent radiocontamination. Duplicate plants for each treatment were harvested, freeze-dried, and autoradiographed as described in section E. The plants were exposed to X-ray film for two weeks.

When translocation of picloram following root absorption was studied, the plants were grown in soil and transferred three days

before treatment to one-half strength Eliasson nutrient solution (47) in glass containers wrapped with aluminum foil. For treatment the plants were further transferred to 250-ml beakers containing 100 ml nutrient solution with 0.4 μCi of ^{14}C-picloram (concentration 0.94 μg/ml). After 1 hr to 4 days the roots of the plants were rinsed under running tap water for 3 minutes. Freeze-killing, drying, and autoradiography were carried out following the procedure outlined in section E, except that the X-ray film exposure time was four weeks for all plants.

II. Quantitative Determination

For quantitative determination of ^{14}C-picloram distribution Canada thistle, soybean, and barley plants were foliarly treated with ^{14}C-picloram in the same way as described above for autoradiography. Four (Canada thistle) or six (soybean or barley) plants made up one replicate and two replicates were used in each treatment. At harvest, 1 to 20 days after treatment, the residue of ^{14}C-picloram on the surface of the treated leaf was washed off with 20 ml of 50 per cent ethanol. The plants were separated into different parts which were stored at -20° C until they were extracted.

The plant parts were ground in 95 per cent ethanol in a Waring blender. The ground materials were kept at room temperature for 12 hr or longer, and then were filtered through Whatman No. 1 filter paper in a Buchner funnel. The residues were extracted again in ethanol overnight. The first and second extracts were combined and concentrated under reduced pressure at 40° - 45° C until nearly dry and then were made up to 5 ml

with 95 per cent ethanol. An aliquot of 0.25 ml from this extract was used for determination of radioactivity by liquid scintillation counting.

J. Metabolism of ^{14}C-Picloram

I. Extraction and Sample Preparation

a. Excised Plant Tissues

Soybean hypocotyls, barley coleoptiles, barley leaf sections, and Canada thistle leaf discs were incubated in ^{14}C-picloram for 1 or 3 days in the manner described in Section H-I. After incubation the sections were washed in distilled water and ground in 95 per cent ethanol in a glass homogenizer. The homogenate was filtered, concentrated under vacuum, and made up to 2 ml with 95 per cent ethanol.

For quantitative determination of ^{14}C-picloram bound to the macromolecules, excised barley leaf sections and Canada thistle leaf discs after incubation in ^{14}C-picloram (in the manner described in Section H-I) were homogenized in a mixture of equal volumes of ice cold 95 per cent ethanol, 90 per cent phenol, and 20 per cent TCA as described by Nooden (117) using 2 ml per gram of tissue. After standing at 2-4° C for 1 hr or more the precipitate was collected by centrifugation and the pellet was washed twice in the above mixture. The washed pellet was dissolved in a small volume of 0.1 M Tris-HCl buffer (pH 7.4), made up to an appropriate volume, and a 0.5 ml aliquot was used for radioactivity determination by liquid scintillation counting.

b. Intact Plants

The plant extracts prepared for quantitative determination of distribution of ^{14}C-picloram (section I-II) were also used for degradation studies. The concentrated plant extracts frequently were too sticky for chromatographic analysis. The crude extracts were purified, therefore, on a florisil column (4 x 25 cm) according to the method of Chang and Vanden Born (32). The extract was evaporated to a small volume, rinsed into the column with chloroform, washed with 200 ml chloroform and 400 ml diethyl ether, and dried by drawing air through the column. The radioactivity then was eluted from the column with 500 ml 95 per cent ethanol. More than 98 per cent of the radioactivity was in the ethanol eluate. The ethanol eluate was concentrated again by evaporating, and part of it was used for chromatographic analysis.

Since picloram or its metabolites may form conjugates with plant constituents (107, 128), the purified extracts were hydrolyzed before identification of the metabolites was carried out. The alcohol in the extract was evaporated and the residue was hydrolyzed with 4N hydrochloric acid for 4 hr on a steam bath. After hydrolysis water was added to bring samples to an appropriate volume and their pH was adjusted to 7.0 for further analysis by paper and thin-layer chromatography.

II. Chromatography

For paper chromatography Whatman No. 1 paper was used and for thin-layer chromatography glass plates coated with silica gel GF.

The solvents used were (a) n-butanol:ammonium hydroxide (28 per cent): water (8:1:1), (b) benzene:propionic acid:water (2:2:1), (c) phenol (90 per cent):water (72:28), and (d) n-butanol:ethanol (95 per cent): water (2:2:1). Solvents (a) and (d) resulted in the best separation both in paper and thin-layer chromatography.

For paper chromatography a small amount of the extract, containing a known amount of radioactivity, was applied as a band on a 4-cm strip of chromatography paper and dried in a stream of warm air. The chromatograms were placed in the chromatography tank for three hours' saturation and then were developed descendingly until the solvent front had advanced 30 cm. Radioactive regions of the chromatograms were detected using a Nuclear Chicago Actigraph III Radiochromatograph Scanner. Autoradiograms were also made on Ilford or Kodak No-screen X-ray film. Exposure time varied with activity level. When more accurate quantitative information was desired, the radioactive zones on the chromatograms, located by reference to the autoradiograms or the scanning results, were cut out and the activity on the paper pieces was assayed in a liquid scintillation counter.

For thin-layer chromatography, 0.25 mm layers of silica gel GF were prepared on 20-cm square glass plates with the Desaga coating apparatus, as described by Stahl (151). In order to obtain uniform coating, the glass plates were thoroughly scrubbed with a scouring powder such as 'Ajax', and then thoroughly brushed under running water, rinsed clean with distilled water, and dried. For five plates, 25 g silica gel was mixed with 50 ml distilled water by vigorous shaking

for 30 to 45 seconds in a stoppered conical flask (250 ml). The slurry was then transferred immediately to the coating apparatus. The coated plates were air-dried overnight, activated in an oven at 110°C for 30 minutes, and stored in a desiccated plate cabinet until used. Plant extracts, 20 to 40 µl depending on the radioactivity, were spotted on the plates with a hypodermic syringe, and dried in a warm air flow from a hair drier. Samples of authentic ^{14}C-picloram were also spotted on the plates. The plates were developed ascendingly for 10 to 12 cm from the origin in a Desaga developing chamber pre-saturated with solvent, and then dried in air. Localization of the radioactive spots was done by autoradiography. For quantitative determination the radioactive spots were carefully scraped from the plates, transferred to scintillation vials, and their activity was determined by liquid scintillation counting.

III. Detection of $^{14}CO_2$

Decarboxylation of ^{14}C-picloram in Canada thistle, soybean, and barley plants was studied by determining the rate of $^{14}CO_2$ liberation from treated plants by the method described by Chow *et al.* (35). Two plants of Canada thistle or four plants of soybean or barley, each treated with 0.1 µCi of ^{14}C-picloram by foliar application (similar to that described for translocation of ^{14}C-picloram, section I-1), were placed in a sealed chamber made from heavy black polyethylene or in a bell-jar wrapped with aluminum foil. The bottom of the jar was sealed to a plastic plate with vaseline. The polyethylene chamber and the bell-jar each contained two strips of filter paper (2.5 x 4 cm) impregnated with hydroxide of hyamine (p-1 diisobutylcresoxyethoxyethyl)-

dimethylbenzylammonium hydroxide). The $^{14}CO_2$ collected on each paper strip was counted in a liquid scintillation counter. In order to minimize artifact effects from starvation of the plants, the plants were replaced with a new set after each two-day period (the used plants were never used again).

RESULTS

A. Susceptibility of Soybean, Canada thistle, and Barley to Picloram

Stem bending and curling and rolling of leaves in soybean and Canada thistle were visible 24 hr after foliage application of picloram at concentrations of 0.125 oz/A or more. Soybean plants were severely injured by 0.063 oz/A of picloram, the lowest dose used in these experiments, and after treatment with 2 oz/A of picloram all the plants died within two weeks (Figure 4). Picloram treatment at 0.063 and 0.125 oz/A prevented the yellowing and subsequent abscission of cotyledonary leaves, usually observed in similar untreated plants three weeks after planting. Canada thistle plants were seriously affected by treatment with 0.25 oz/A of picloram, but plants died completely in two weeks only after treatment with 8 oz/A or more picloram. The rates of application which caused death of soybean and Canada thistle plants caused no significant injury symptoms to barley. No barley plants died in the two-week period at a dose as high as 64 oz/A, although serious injury symptoms (stunting of plants, stem bending, and leaf rolling) were observed at dosages of 16 oz/A or more. In the present experiments typical malformations in picloram-treated plants included twisting and swelling of stems, leaf curling, and death of growing point (in Canada thistle and soybean). These symptoms are similar to those observed by other workers for picloram (34, 52, 63, 100, 162) and other auxin-herbicides (30, 173) in many broadleaf and grass species.

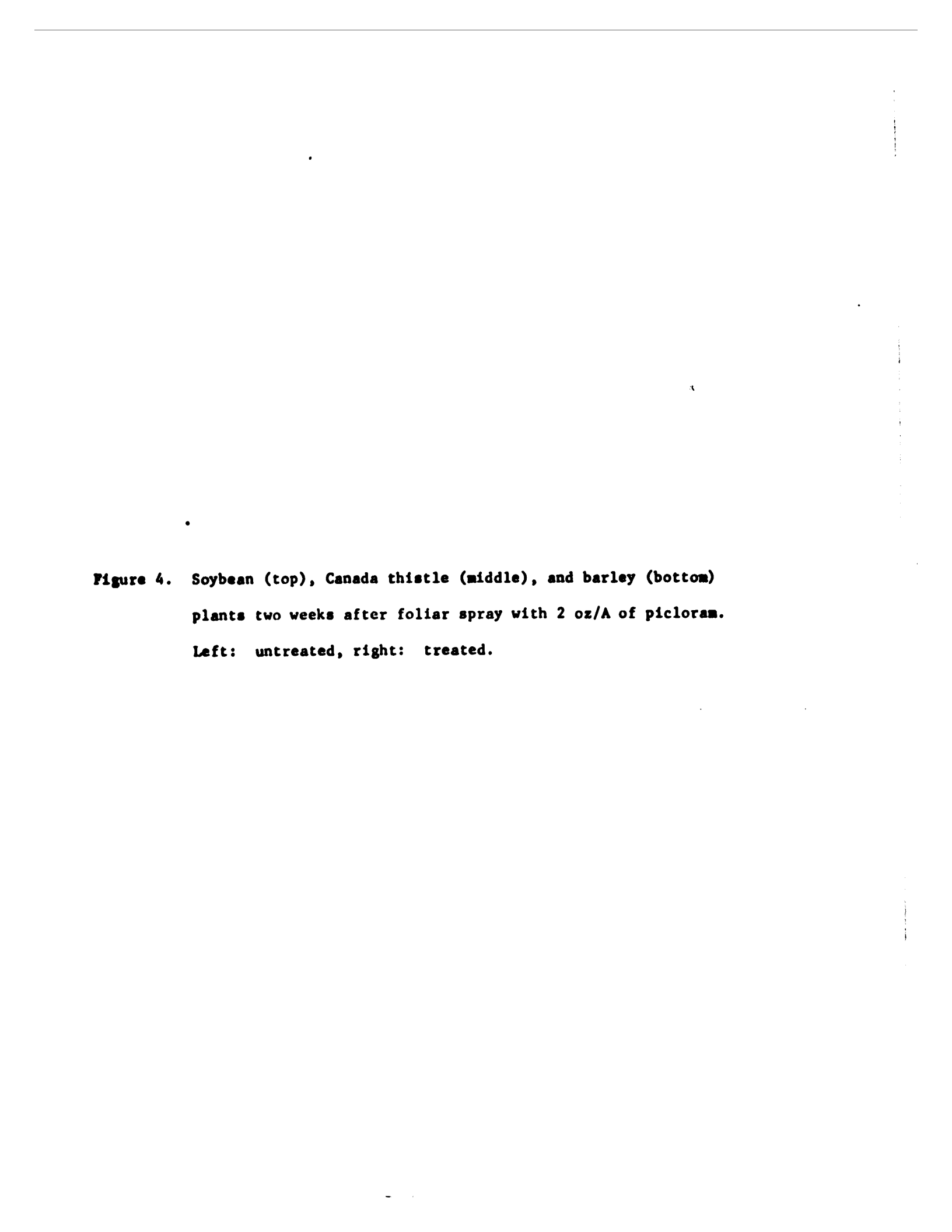
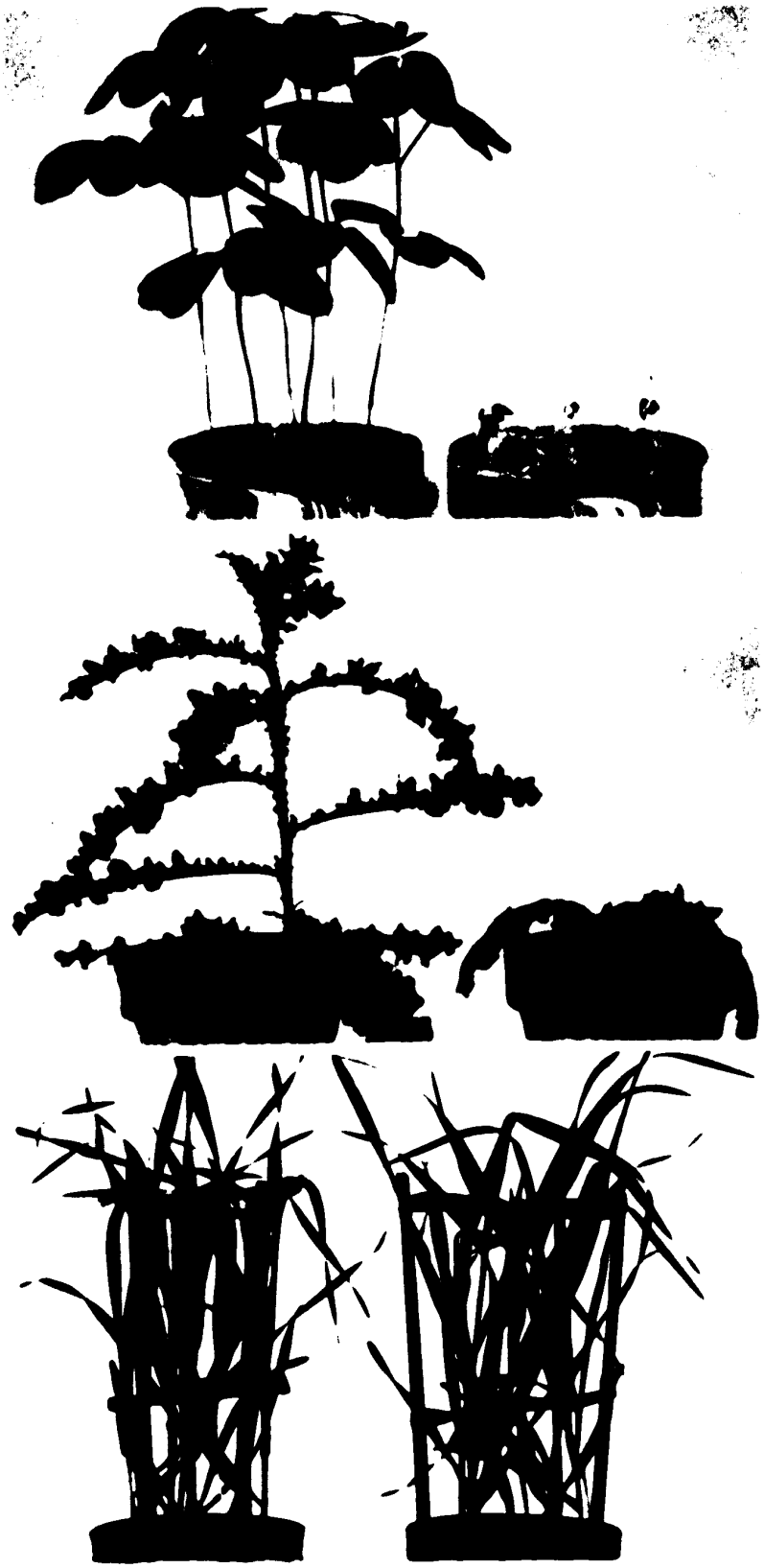


Figure 4. Soybean (top), Canada thistle (middle), and barley (bottom) plants two weeks after foliar spray with 2 oz/A of picloram. Left: untreated, right: treated.



The effect of picloram treatment on the growth of these three species is illustrated in Figure 5, in which the dry weight of the plants, expressed as a percentage of the untreated control, is plotted against the rates of application of the herbicide. From the graph, ED_{50} values (dose required to reduce growth by half) for these species were estimated. These values were 0.23 oz/A for soybean, 0.26 oz/A for Canada thistle, and 52 oz/A for barley, under the experimental conditions in the greenhouse. The results confirm that soybean is very susceptible to picloram, Canada thistle moderately susceptible, and barley relatively resistant. Ultimate grain yield is not taken into account in these experiments but must be considered under field conditions (162, 171).

B. $^{14}CO_2$-Fixation by Excised Leaves

I. Relative Rates of $^{14}CO_2$-Fixation

Photosynthetic $^{14}CO_2$-fixation by excised leaves of Canada thistle and soybean was reduced significantly ($P = 0.01$) by all concentrations of picloram used to treat the source plants prior to leaf excision (Table 2). At 10 µg/ml the inhibition was about 50 per cent. Soybean leaves from plants treated with 20 µg/ml picloram were distorted (rolled) badly, and were not used in $^{14}CO_2$-fixation studies. In corn a small but significant reduction in fixation occurred only at 20 µg/ml, the highest concentration of picloram used.

None of the picloram treatments in this study completely inhibited photosynthetic $^{14}CO_2$-fixation (light fixation). This observation agrees with the findings of other investigators (8, 9, 40) in work

Figure 5. Effect of foliar spray of picloram on growth, measured as dry weight, of soybean (So), Canada thistle (CT), and barley (Ba) plants. Picloram concentration is plotted on a log scale. The plants were grown in the greenhouse, and were harvested two weeks after treatment.

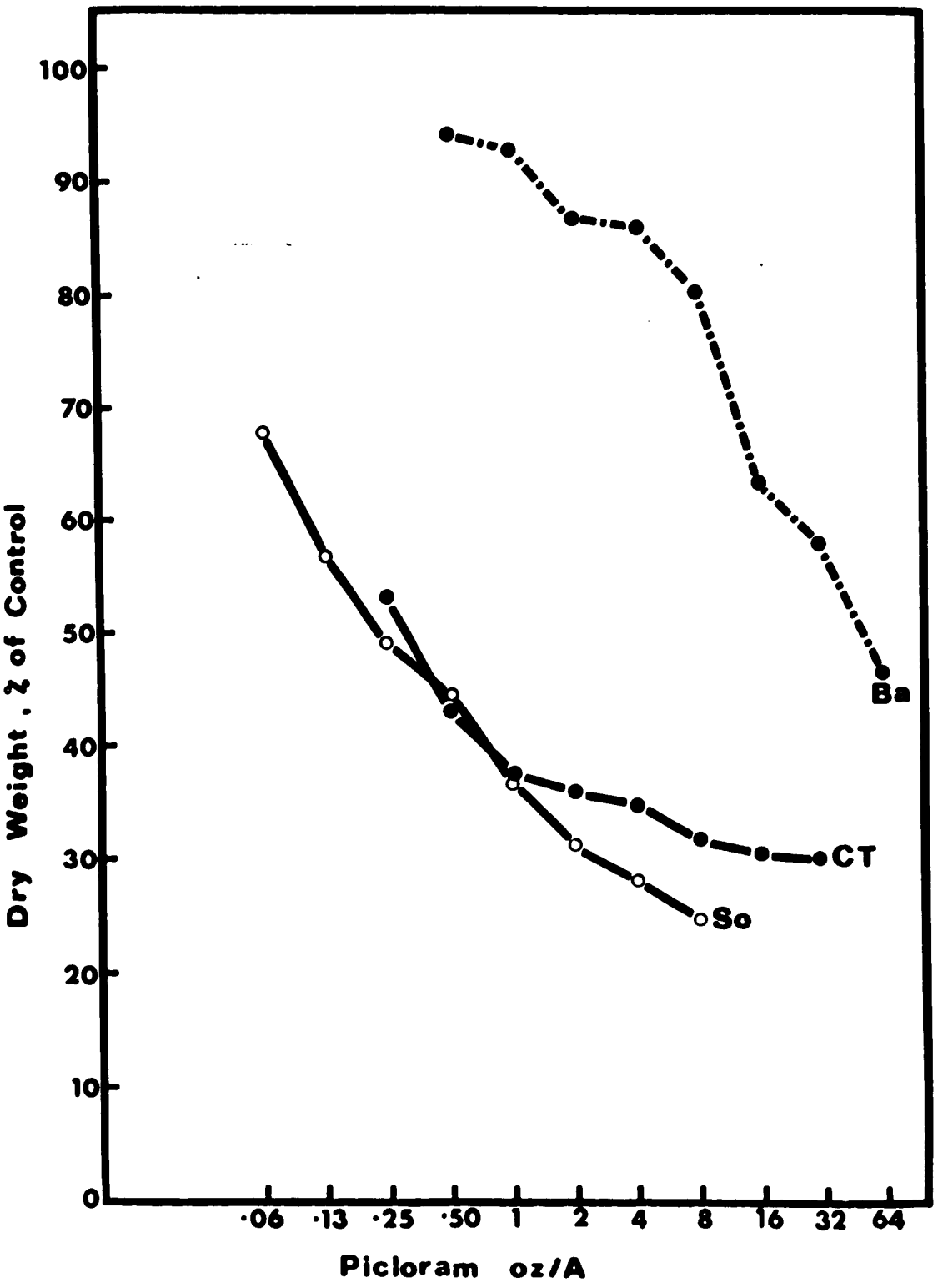

100
90
80
70
60
50
40
30
20
10
0
Dry Weight, % of Control
Ba
CT
So
·06
·13
·25
·50
1
2
4
8
16
32
64
Picloram oz/A

TABLE 2

Effect of root-absorbed picloram on light-fixation of $^{14}CO_2$ by excised leaves of Canada thistle, soybean, and corn, expressed as total radioactivity in the ethanol-soluble fraction[a]

Picloram conc. µg/ml	Canada thistle		Soybean		Corn	
	10^{-3} x dpm[b]	%[c]	10^{-3} x dpm[b]	%[c]	10^{-3} x dpm[b]	%[c]
0	4235[d]		6804		7728	
1	3671	87	5379	79	7399	96
0	4166		6804		5462	
10	1923	46	3849	57	5126	94
0	6250				4810	
20	2052	33			4187	87

[a] Excised leaves (one for each concentration) exposed to about 1.0 mCi $^{14}CO_2$ for 30 min. at 23 ± 0.5° C under 10220 lux fluorescent light at leaf level.

[b] Dpm/cm^2 of leaf surface; average of duplicates.

[c] Expressed as per cent of corresponding control.

[d] L.S.D. (P = 0.01): Canada thistle, 423,000 dpm; soybean, 413,986 dpm; corn, 395,073 dpm.

with s-triazine, phenylurea, and uracil herbicides. Complete blockage of the Hill reaction would conceivably block all CO_2-fixation, provided this is the only energy source and the only CO_2-fixation pathway. In the present study either picloram did not reach the active sites in the chloroplasts in sufficient amounts to bring about complete inhibition, or some fixation occurred via a picloram-insensitive pathway. An examination of picloram effects on dark $^{14}CO_2$-fixation in the three species showed significant inhibition ($P = 0.05$) only in soybean, at 10 µg/ml (Table 3). In Canada thistle and corn no inhibition occurred.

II. Distribution of ^{14}C after $^{14}CO_2$-Fixation

Since there was no marked effect of picloram on dark $^{14}CO_2$-fixation only the ethanol extracts from light fixation studies were analyzed (Table 4). Inhibition of $^{14}CO_2$-fixation in Canada thistle and soybean leaves as a result of picloram treatment (Table 2) was accompanied by marked reductions in the amounts of labeled sucrose and alanine and by increases in labeled malic acid, aspartic acid, glutamic acid, asparagine, and serine. In corn the only effect of picloram on distribution of ^{14}C was a small decrease in the relative amount of labeled sucrose after treatment with 20 µg/ml, the highest concentration used.

C. Translocation of ^{14}C-Assimilates

Autoradiograms in Figures 6, 7, and 8 show the distribution of ^{14}C-assimilates in picloram-treated Canada thistle, soybean, and corn

TABLE 3

Effect of root-absorbed picloram on dark-fixation of $^{14}CO_2$ by excised leaves of Canada thistle, soybean, and corn, expressed as total radioactivity in the ethanol-soluble fraction[a].

Picloram conc., µg/ml	Canada thistle		Soybean		Corn	
	10^{-3} x dpm[b]	%[c]	10^{-3} x dpm[b]	%[c]	10^{-3} x dpm	%[c]
0	12.7		39.1		50.9	
1	13.0	102	37.8	97	47.2	93
0	12.2		26.4		40.6	
10	13.1	107	22.6	86[d]	37.8	93
0	15.1				40.2	
20	14.5	96			37.1	92

[a] Excised leaves (one for each concentration) exposed to about 1.0 mCi $^{14}CO_2$ for 1 hr at 20°C in complete darkness.

[b] Dpm/cm^2 of leaf surface; average of duplicates.

[c] Expressed as per cent of corresponding control.

[d] Difference from control exceeds the $P_{0.05}$ level of significance.

TABLE 4

Effect of picloram on relative amounts of ^{14}C-labeled compounds following light fixation of $^{14}CO_2$. Data are expressed as per cent of total radioactivity in the ethanol-soluble fraction.[a]

Compound	Canada thistle				Soybean			Corn			
	Picloram concentration, ppm										
	0	1	10	20	0	1	10	0	1	10	20
Sucrose	52.0	46.3	31.4	27.2	38.8	25.2	19.8	56.3	58.1	50.0	46.8
Asparagine	9.9	10.3	14.2	15.3	8.7	13.7	13.6	2.7	2.9	3.2	3.0
Malic acid	10.2	12.4	14.3	13.9	14.0	17.4	20.7	16.6	14.8	16.9	17.4
Aspartic acid	3.3	5.6	6.9	7.4	4.2	9.0	8.6	2.7	3.4	4.3	4.9
Glutamic acid	4.3	3.5	6.3	8.3	3.1	3.2	4.8	2.2	2.1	3.0	2.9
Glycine	3.9	3.9	5.0	4.9	3.6	4.6	4.1	1.1	1.7	2.2	2.4
Serine	3.1	5.2	6.9	7.6	9.2	11.2	12.2	2.4	2.7	3.9	4.6
Alanine	3.4	2.6	1.8	1.8	6.5	3.5	2.9	10.0	8.8	11.3	11.5
Fumaric acid	2.4	1.9	2.6	3.1	-	-	-	-	-	-	-
Fructose	0.9	0.7	1.2	1.3	1.2	0.6	0.8	1.1	0.9	0.9	1.3
Sugar Phosphates	2.6	4.1	4.5	4.4	3.0	3.0	4.7	1.8	2.2	2.0	2.3
Misc. unknown	4.0	3.5	4.9	4.8	7.7	8.6	7.8	3.1	3.4	2.7	2.9

[a] Excised leaves (one for each concentration) exposed to 1.0 mCi $^{14}CO_2$ for 30 min at 23 ± 0.5° C under 10220 lux fluorescent light at leaf level. Control values are means of four leaves; all other values are means of duplicates.

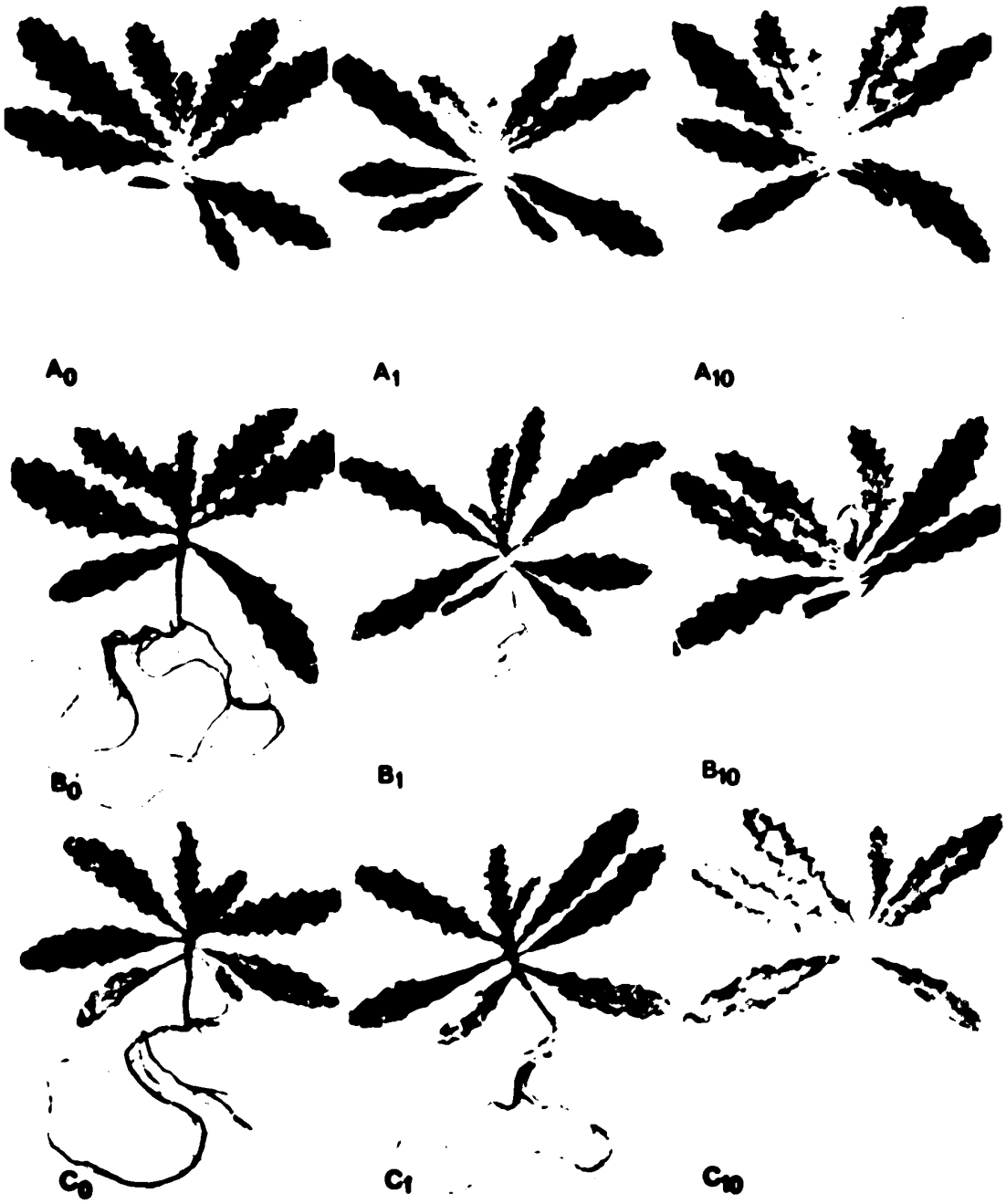
plants, respectively, after exposure to $^{14}CO_2$.

Inhibition of $^{14}CO_2$-fixation in Canada thistle as a result of picloram treatment (Figure 6, A series) agrees with the count data on excised leaves (Table 2). The areas of greatest inhibition as shown in the autoradiograms do not completely correspond, however, to distribution patterns of ^{14}C-labeled picloram in Canada thistle plants after root uptake (146). For example, the radioactivity from ^{14}C-picloram after root uptake was concentrated at the leaf margins, whereas the inhibition of $^{14}CO_2$-fixation in picloram-treated plants was greatest near the midvein of the leaves. However, $^{14}CO_2$-fixation by young growing leaves of Canada thistle, where ^{14}C-picloram was accumulated after root uptake, was greatly inhibited by picloram treatment.

Translocation of ^{14}C-assimilates to roots of Canada thistle plants was reduced markedly by 1 µg/ml picloram, and stopped completely by 10 µg/ml. Translocation to young leaves was inhibited nearly completely by 10 µg/ml picloram, but the 1 µg/ml concentration did not appear to affect this transport. The effect of picloram on distribution of ^{14}C-assimilates evidently is not strictly one of sink-disturbance in the young growing tissues, because the assimilate source is also seriously affected.

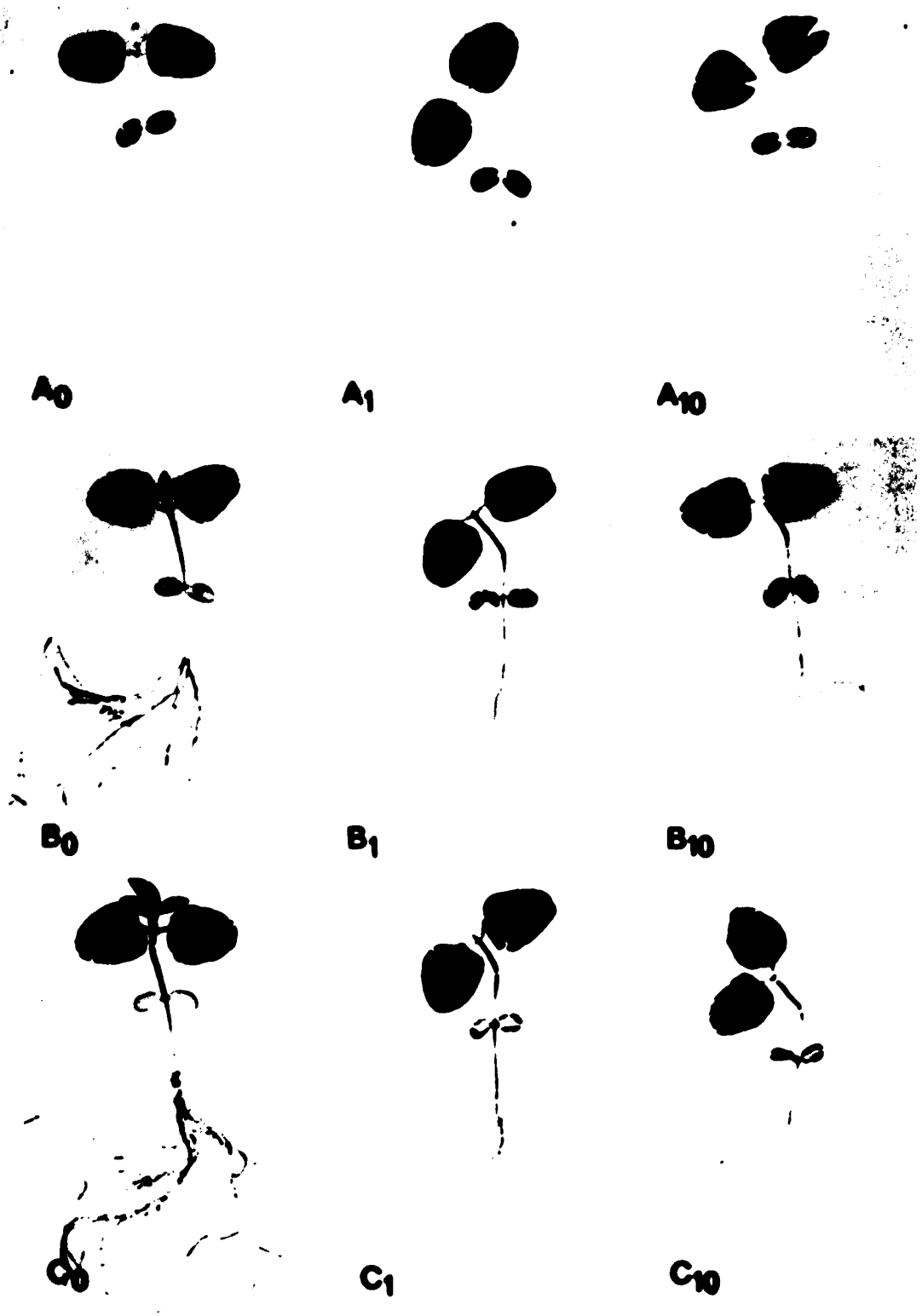
In contrast with Canada thistle results, autoradiograms of soybean plants showed little evidence of picloram-induced inhibition of $^{14}CO_2$-fixation (Figure 7, A series). Transport of assimilates to young leaves (where very little $^{14}CO_2$ was fixed) was blocked completely by picloram at 1 µg/ml, but translocation to roots was only partly blocked

Figure 6. Distribution of ^{14}C-assimilates in Canada thistle plants (A) 0 hr, (B) 4 hr, (C) 24 hr after exposure to $^{14}CO_2$ for 10 min. Before exposure to $^{14}CO_2$ the plants were treated for 24 hr with 100 ml of 0, 1, or 10 µg/ml picloram in nutrient solution.


A0
A1
A10
B0
B1
B10
C0
C1
C10


A0
A1
A10
B0
B1
B10
C0
C1
C10

Figure 7. Distribution of ^{14}C-assimilates in soybean plants (A) 0 hr, (B) 4 hr, (C) 24 hr after exposure to $^{14}CO_2$ for 10 min. Before exposure to $^{14}CO_2$ the plants were treated for 24 hr with 100 ml of 0, 1, or 10 µg/ml picloram in nutrient solution.


A0
A1
A10
B0
B1
B10
C0
C1
C10

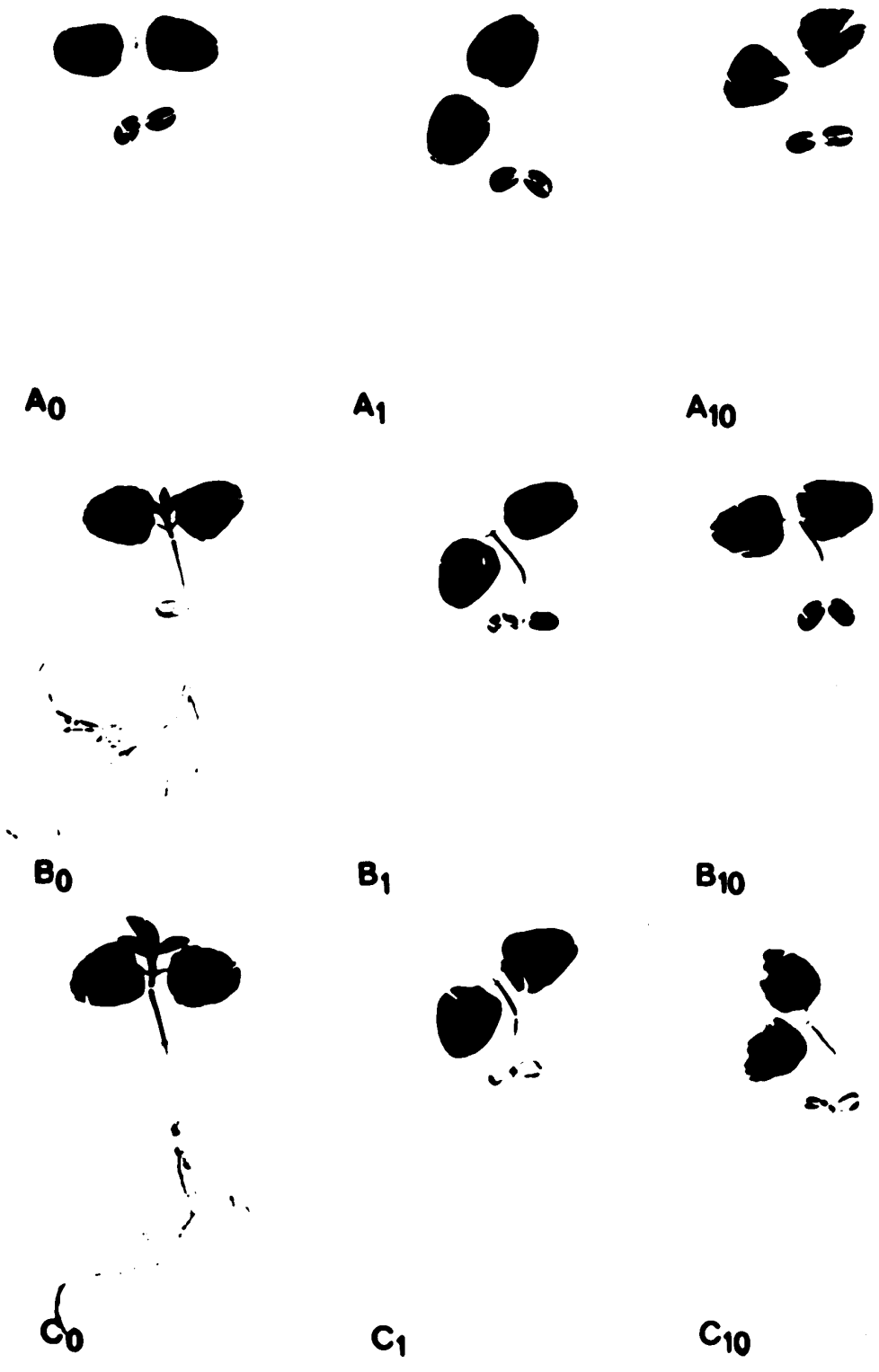
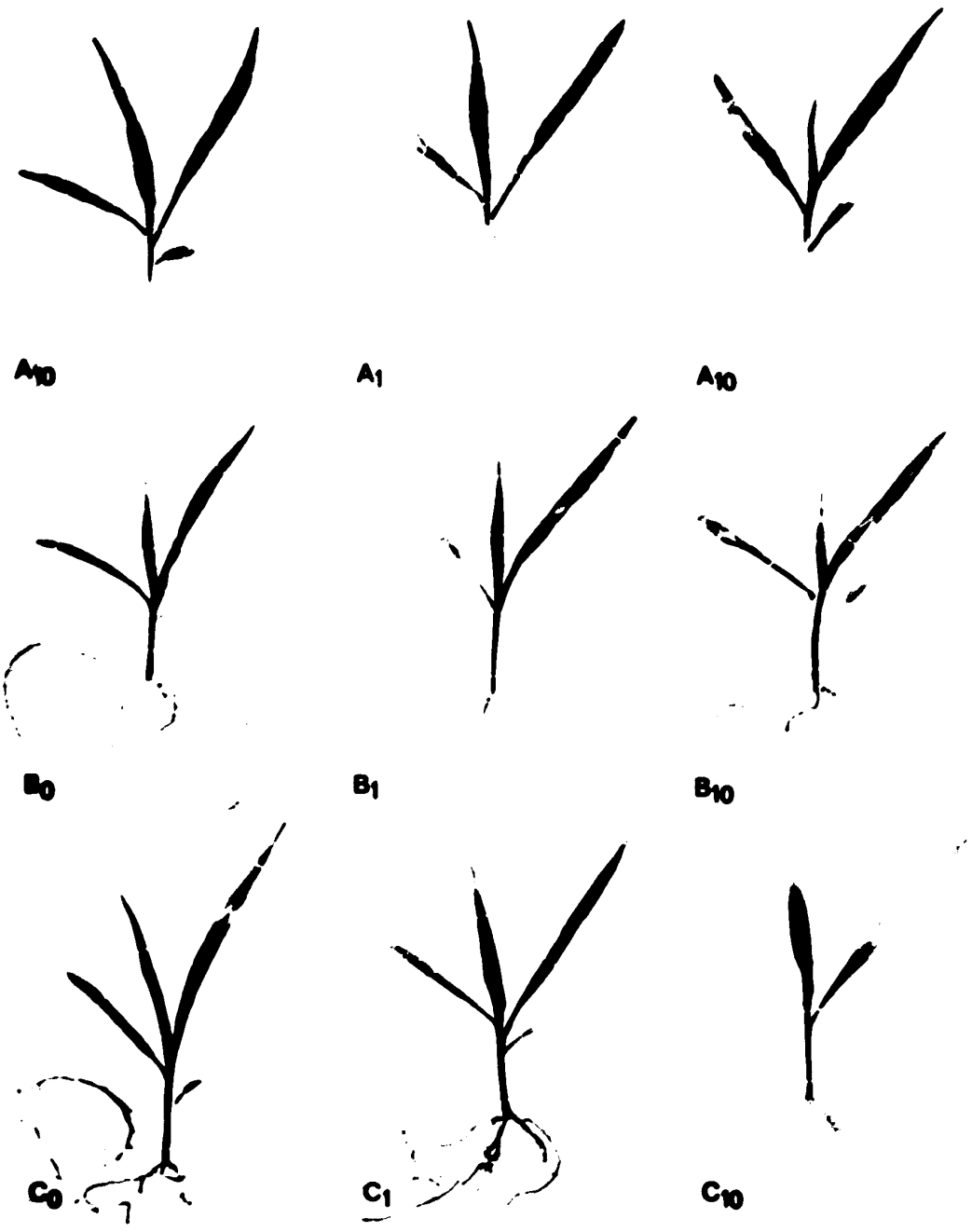
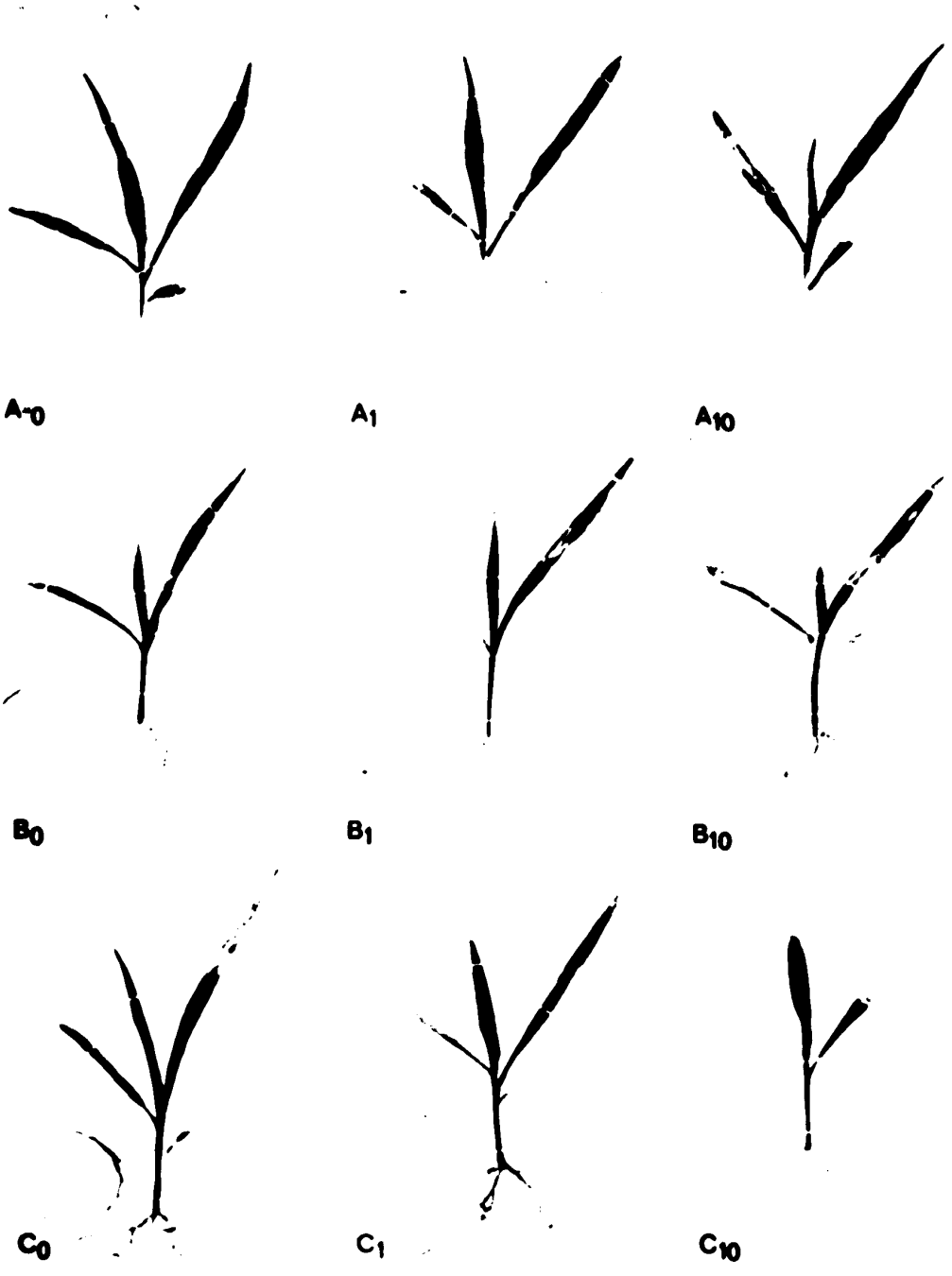

A0
A1
A10
B0
B1
B10
C0
C1
C10

Figure 8. Distribution of ^{14}C-assimilates in corn plants (A) 0 hr, (B) 4 hr, (C) 24 hr after exposure to $^{14}CO_2$ for 10 min. Before exposure to $^{14}CO_2$ the plants were treated for 24 hr with 100 ml of 0, 1, or 10 µg/ml picloram in nutrient solution.


A10
A1
A10
B0
B1
B10
C0
C1
C10


A-0
A1
A10
B0
B1
B10
C0
C1
C10

even at 10 µg/ml. Cotyledonary leaves in control plants in 24 hr exported nearly all the ^{14}C fixed by them, and this export was reduced sharply by picloram treatment. Accumulation of activity in the first internode, presumably exported downward from the primary leaves, was reduced but not stopped by picloram.

Translocation of ^{14}C-assimilates in corn seedlings was not noticeably affected by picloram (Figure 8). At 10 µg/ml some inhibition of transport to the roots occurred, but only after a 24-hr transport period.

D. Chlorophyll, RNA, and Protein Levels of Intact Plants

Chlorophyll content of soybean and Canada thistle plants was markedly reduced by picloram both one and three days after treatment (Table 5). Three days after treatment the reduction was about 25 per cent. The small reduction in chlorophyll content in barley three days after treatment was not statistically significant.

RNA and protein levels of picloram-treated soybean and Canada thistle plants were significantly higher ($P = 0.01$) than those in similar untreated plants (Tables 6 and 7). In soybean, for instance, RNA increased by 24 to 27 per cent and protein by 20 to 24 per cent one or three days after picloram treatment. Increases in RNA and protein in picloram-treated Canada thistle were slightly lower than those in soybean plants. In barley no significant increases in RNA and protein were detected.

TABLE 5

Effect of picloram on chlorophyll content (mg/g dry weight) of soybean, Canada thistle, and barley plants.[a]

Treatment	Soybean		Canada thistle		Barley	
	1 day	3 days	1 day	3 days	1 day	3 days
Control	7.6	7.4	6.8	6.7	7.9	8.1
Picloram	6.6	5.3	6.2	5.2	7.7	7.6
%[c]	87	72	91	77	98	94

[a] Plants sprayed with 0.05 M phosphate buffer (pH 6.0) containing 1 per cent Atlox 210, without or with 250 µg/ml picloram. Plants harvested one or three days after spraying, freeze-dried, and ground prior to chlorophyll determination.

[b] L.S.D.: 0.6 mg (P = 0.05); 0.9 mg (P = 0.01).

[c] Expressed as per cent of corresponding control.

TABLE 6

Effect of picloram on RNA content (mg/g dry weight) of soybean, Canada thistle, and barley plants.[a]

Treatment	Soybean		Canada thistle		Barley	
	1 day	3 days	1 day	3 days	1 day	3 days
Control	16.6[b]	18.1	11.4	11.8	14.0	14.4
Picloram	21.1	22.4	14.1	14.4	14.4	14.9
%[c]	127	124	124	122	103	104

a Plants sprayed with 0.05 M phosphate buffer (pH 6.0) containing 1 per cent Atlox 210, without or with 250 µg/ml picloram. Plants harvested one or three days after spraying, freeze-dried, and ground prior to RNA determination.

b L.S.D.: 1.6 mg (P = 0.05); 2.1 mg (P = 0.01).

c Expressed as per cent of corresponding control.

TABLE 7

Effect of picloram on protein content (mg/g dry weight) of soybean, Canada thistle, and barley plants.[a]

Treatment	Soybean		Canada thistle		Barley	
	1 day	3 days	1 day	3 days	1 day	3 days
Control	161	187	116	123	142	150
Picloram	200	222	138	141	154	159
%[c]	124	120	119	116	108	106

a Plants sprayed with 0.05 M phosphate buffer (pH 6.0) containing 1 per cent Atlox 210, without or with 250 µg/ml picloram. Plants harvested one or three days after spraying, freeze-dried, and ground prior to protein determination.

b L.S.D.: 13.5 mg (P = 0.05); 18.2 mg (P = 0.01).

c Expressed as per cent of corresponding control.

E. Effect of Picloram on RNA and Protein Metabolism of Excised Plant Tissues

I. RNA and Protein Content

The RNA content (µg/section) of excised soybean hypocotyls, barley coleoptiles, barley leaf sections, and Canada thistle leaf discs decreased by 20 to 30 per cent during an 8-hr incubation period (Table 8). During the same incubation period, protein content of these tissues decreased by only 7 to 16 per cent. Similar losses in RNA and protein after incubation have been observed in excised cucumber hypocotyl (173), corn mesocotyl (81), pea stem sections (50), soybean hypocotyl (82, 85), and excised leaves from a number of different plant species (cf 159). The development of enzymes such as ribonuclease and protease and lack of supply of growth hormones are possible reasons for this loss of RNA and protein of excised plant tissues.

The response of excised soybean hypocotyl, barley coleoptile, barley leaf sections, and Canada thistle leaf discs to picloram varied with the tissue and the concentration of picloram involved. The net loss of RNA and protein in soybean hypocotyl sections was considerably reduced by picloram, both at growth-promoting (10 µg/ml) and growth-inhibiting (500 µg/ml) concentrations (Table 8). Thus, picloram caused an overall increase in RNA and protein content relative to control sections. Increases in RNA and protein concentration were greater at 10 µg/ml (51 and 44 per cent, respectively) than at 500 µg/ml picloram (19 and 12 per cent, respectively). Picloram at 10 µg/ml increased RNA and protein contents of soybean hypocotyl sections

TABLE 8

Changes in RNA and protein content of excised soybean hypocotyls, barley coleoptiles, barley leaf sections, and Canada thistle leaf discs in response to picloram.[a]

Tissue	Picloram, µg/ml	Per cent increase in fresh weight[b]	RNA µg/section[b]	RNA %[c]	Protein µg/section[b]	Protein %[c]
Soybean hypocotyls	Initial		80		668	
	0	35	63		602	
	10	51	95	151	866	144
	500	4	72	119	675	112
Barley coleoptiles	Initial		28		228	
	0	36	21		189	
	10	94	22	105	204	108
	500	19	25	119	224	119
Barley leaf sections	Initial		20		129	
	0	5	14		117	
	10	6	14	100	121	103
	500	5	16	114	125	107
Canada thistle leaf discs	Initial		31		267	
	0	6	25		247	
	10	8	27	108	264	107
	500	3	26	104	235	95

[a] Excised tissues were incubated at 25°C for 8 hr in 0.01 M phosphate buffer (pH 6.0) containing 1 per cent sucrose, 20 µg/ml streptomycin sulfate, and different concentrations of picloram.

[b] All values in the table are means of three different experiments.

[c] Expressed as per cent of corresponding control.

over the initial levels suggesting that it not only decreased RNA and protein loss but also increased the synthesis of new RNA and protein. If RNA and protein contents were calculated on the basis of final fresh weight of the tissue (data not shown) the increase was greater at growth-inhibiting than at growth-promoting concentration of picloram. This is mainly because of a drastic reduction in the fresh weight with only a small reduction in RNA and protein content of the tissue after incubation in picloram at 500 µg/ml, relative to control values. It should be emphasized that the term 'increase' is used relative to the RNA and protein levels in excised tissues incubated in buffer alone. While, for example, after some hours of treatment picloram brought about increases in RNA and protein levels relative to buffer controls, relative to initial levels it only decreased losses.

In barley coleoptiles and barley leaf sections, in contrast to soybean hypocotyls, picloram at 10 µg/ml had little effect on the net loss of RNA and protein, but at the same time it did not stimulate RNA and protein synthesis either, even though in barley coleoptiles growth was more than double that in control sections (Table 8). It is quite possible that picloram at a growth-promoting concentration stimulated the synthesis of new RNA and protein and at the same time promoted the loss of RNA and protein thus resulting in no net change in RNA and protein content of barley coleoptile and barley leaf sections. Picloram at a growth-inhibiting concentration, on the other hand, decreased the net loss of RNA and protein and thus caused an increase (19 per cent over the controls in barley coleoptiles) in RNA and protein content.

Canada thistle leaf discs showed only a small increase in RNA and protein contents (8 and 7 per cent, respectively) when incubated with picloram at 10 µg/ml (Table 8). No marked effect on RNA and protein levels was observed at 500 µg/ml picloram, except that there was a small increase in RNA and a decrease in protein content relative to corresponding controls.

From the data presented in Table 8 it is obvious that there is no strict correlation between the effects of picloram on growth and on RNA and protein content of excised sections. For example, in soybean hypocotyl picloram at 500 µg/ml resulted in about 88 per cent reduction in fresh weight but an increase in RNA and protein content. Similarly, in barley coleoptile, picloram at 10 µg/ml caused a large increase in fresh weight but nearly no effect on RNA and protein content.

The effects of picloram on RNA and protein metabolism in excised plant tissues described above may be explained either in terms of an effect on RNA and protein synthesis or an effect on the metabolic breakdown of RNA and protein. The experiments described in the next section were designed to help differentiate between these possibilities.

II. Effect of Picloram on Time-Course of Growth and RNA and Protein Biosynthesis

The influence of different concentrations of picloram on growth and on the incorporation of ^{14}C-labeled precursors into newly synthesized RNA and protein by excised soybean hypocotyl and barley coleoptile sections over a 24-hr period was investigated. The results

are illustrated in Figures 9, 10, and 11.

Both soybean hypocotyl and barley coleoptile sections increased in fresh weight when incubated in buffer for periods of up to 24 hr (Figure 9). Addition of picloram at 10 µg/ml to the incubation medium greatly enhanced such growth. An increase in growth as high as 187 per cent over the control was obtained in barley coleoptile sections after 6 hr incubation in buffer containing 10 µg/ml picloram. Picloram at 100 µg/ml enhanced the growth of soybean hypocotyl sections for the first 6 hr of incubation but after that inhibited their growth. No such inhibitory effect of picloram at 100 µg/ml occurred in barley coleoptile sections. Picloram at 500 µg/ml drastically inhibited the growth of both soybean hypocotyl and barley coleoptile sections throughout the experiment, except for a small but reproducible stimulation of barley coleoptile growth during the first 2 hr. Comparisons of fresh weight and length confirmed that growth in soybean hypocotyl and barley coleoptile sections was due mainly to an increase in cell elongation rather than to nonpolar swelling of the tissues. The enhancement of growth of hypocotyl and coleoptile by picloram in my experiments is similar to that reported by Eisinger and Morré (46), Kefford and Caso (79), and Schrank (140) who observed a large increase in growth (fresh weight and length) of oat and wheat coleoptile and soybean hypocotyl sections by picloram at concentrations ranging from 2.5 to 25 µg/ml.

Incorporation of ^{14}C-8-ATP into RNA by control sections (incubated in buffer without picloram) varied from 8 to 35 per cent of the total ^{14}C absorbed and that of ^{14}C-U-leucine into protein from 17 to 59 per cent, during a 24-hr incubation period. After an initial

Figure 9. Effect of picloram on time-course of growth, as per cent increase in fresh weight over the initial weight, of soybean hypocotyl (upper) and barley coleoptile (lower) tissue. Excised sections were incubated in the dark at 25°C in 5 ml of 0.01 M phosphate buffer (pH 6.0) containing 1 per cent sucrose, 20 µg/ml streptomycin sulfate, and 0(A), 10(B), 100(C), and 500(D) µg/ml picloram. Data plotted are means of three experiments.

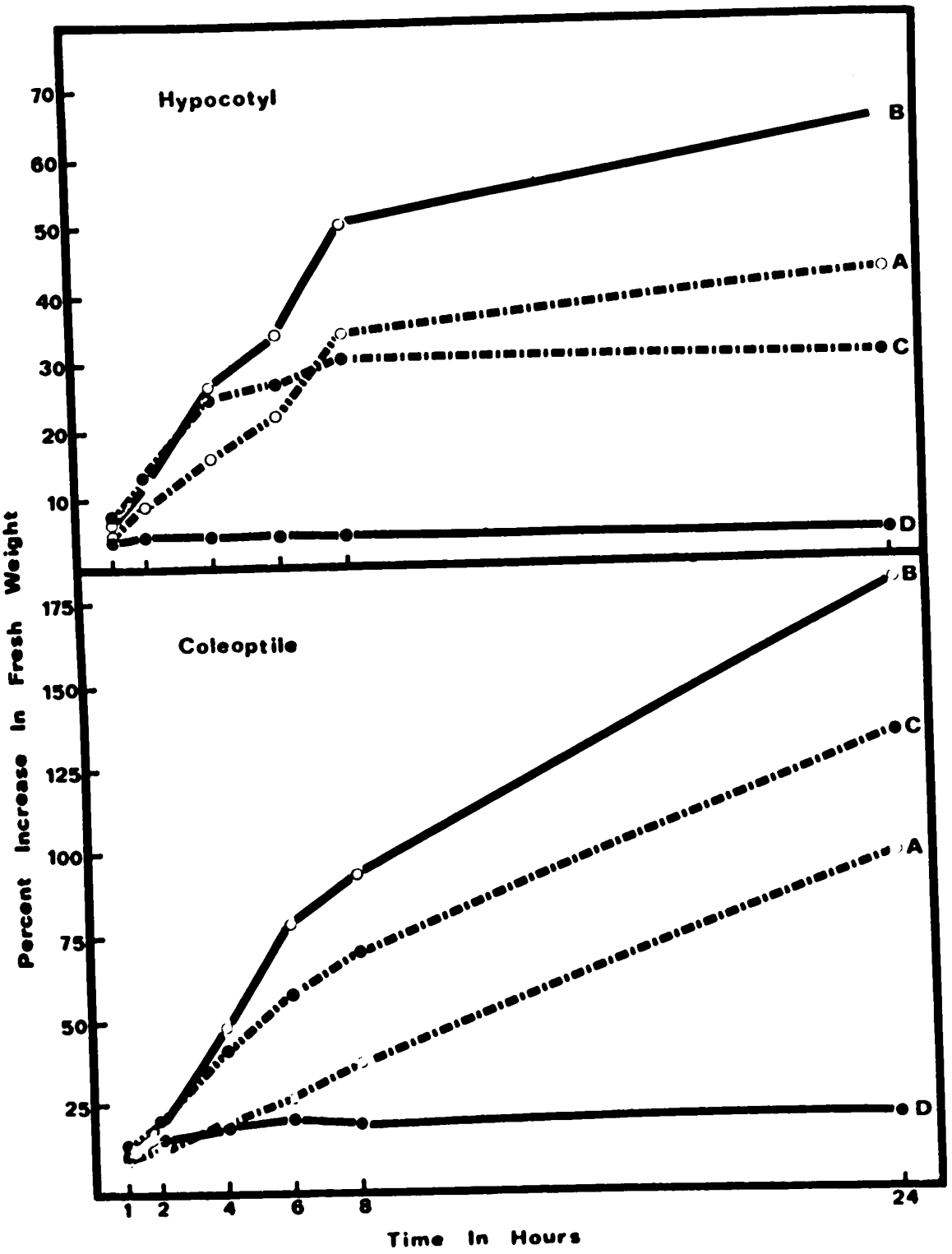
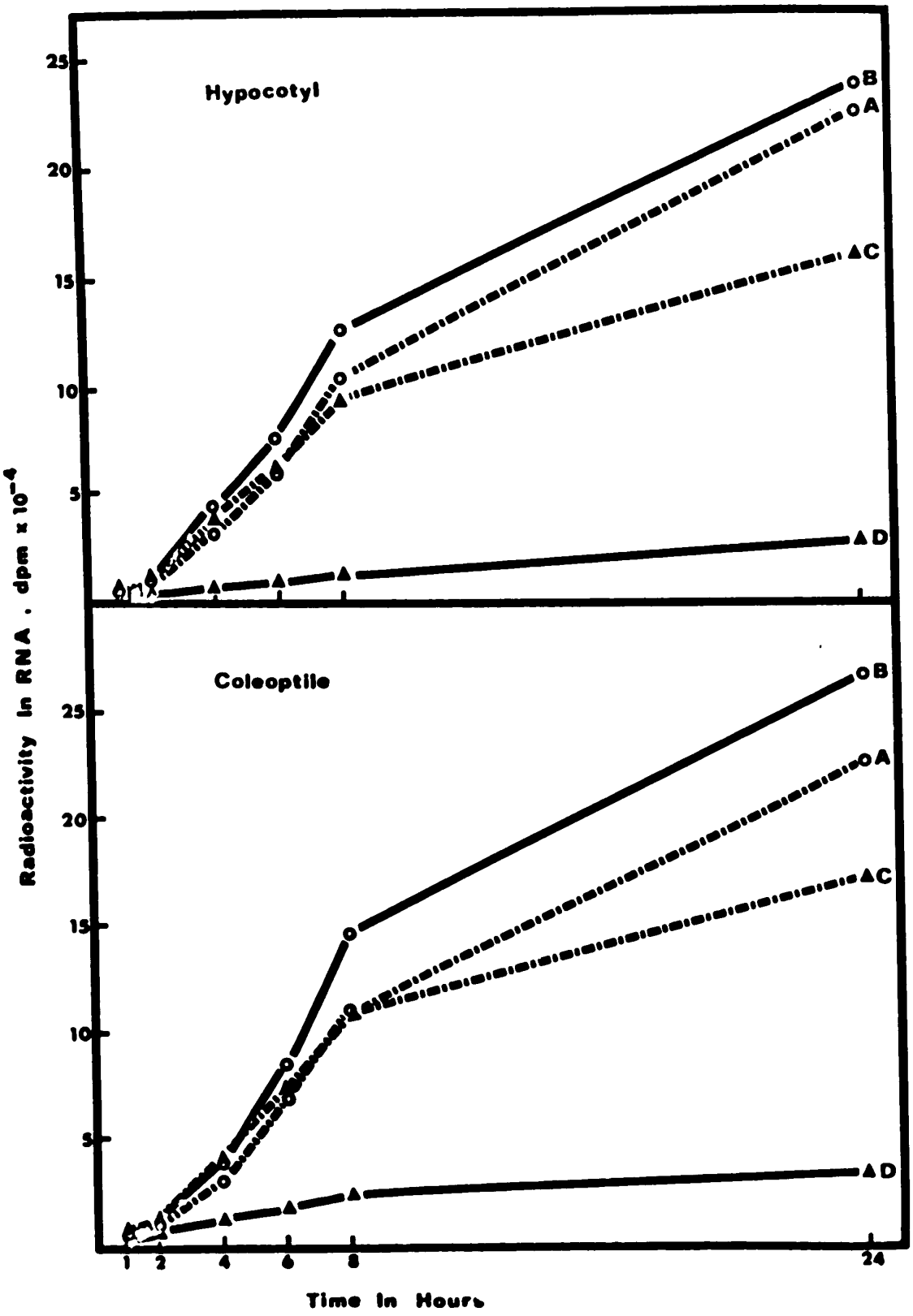

Hypocotyl
Coleoptile
B
A
C
D
70
60
50
40
30
20
10
175
150
125
100
75
50
25
1
2
4
6
8
24
Percent Increase In Fresh Weight
Time In Hours

Figure 10. Effect of picloram on time-course of RNA biosynthesis, measured as incorporation of ^{14}C-8-ATP, in soybean hypocotyl (upper) and barley coleoptile (lower) sections. Excised sections were incubated in the dark at 25°C in 5 ml of 0.01 M phosphate buffer (pH 6.0) containing 1 per cent sucrose, 20 µg/ml streptomycin sulfate, and 0(A), 10(B), 100(C), and 500(D) µg/ml of picloram. ^{14}C-8-ATP was added at 0.1 µCi per ml of incubation medium. Radioactivity in RNA is represented as dpm/g initial fresh weight of tissue. Data plotted are means of three experiments.


Hypocotyl
Coleoptile
B
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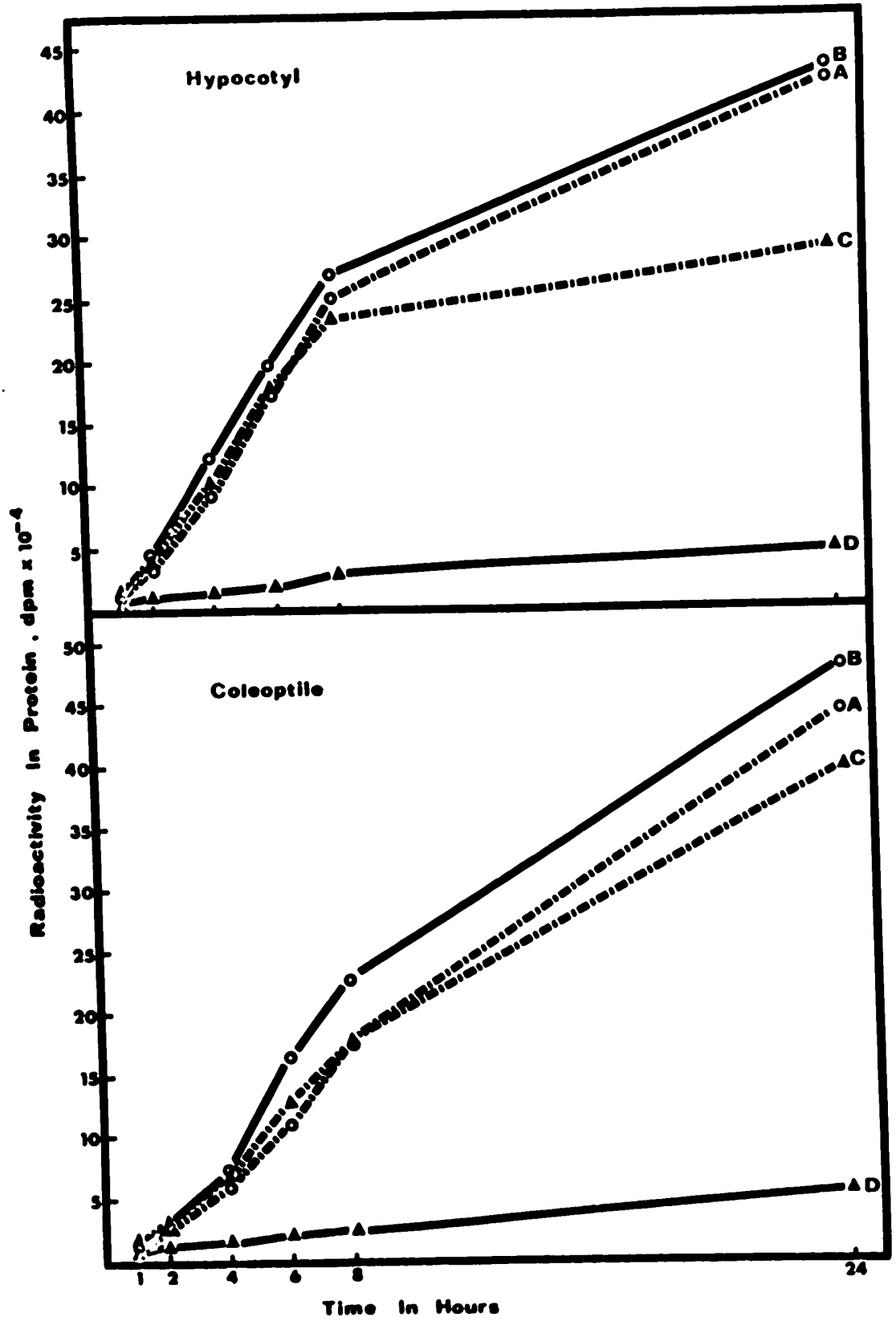
Radioactivity in RNA . dpm x 10⁻⁴
Time in Hours

Figure 11. Effect of picloram on time-course of protein biosynthesis, measured as incorporation of ^{14}C-U-leucine, in soybean hypocotyl (upper) and barley coleoptile (lower) sections. Excised sections were incubated in the dark at 25°C in 5 ml of 0.01 M phosphate buffer (pH 6.0) containing 1 per cent sucrose, 20 µg/ml streptomycin sulfate, and 0(A), 10(B), 100(C), and 500(D) µg/ml of picloram. ^{14}C-U-leucine was added at 0.05 µCi per ml of incubation medium. Radioactivity in protein is represented as dpm/g initial fresh weight of tissue. Data plotted are means of three experiments.


Hypocotyl
Coleoptile
B
A
C
D
Radioactivity in Protein, dpm x 10^{-4}
Time in Hours
1
2
4
6
8
24

lag of about 2 hr the synthesis of new RNA (incorporation of ^{14}C-ATP), both in control and picloram-treated (10 and 100 µg/ml) tissue, proceeded at a nearly linear rate for at least 8 hr (Figure 10). No such lag period was observed for protein synthesis (incorporation of ^{14}C-leucine) which proceeded at an approximately linear rate for up to 8 hr of incubation (Figure 11). Picloram at 10 µg/ml enhanced the biosynthesis of RNA and protein (incorporation of ^{14}C precursors) over that in controls both in soybean hypocotyl and barley coleoptile sections. The increase was rather small (not statistically significant except for ATP and leucine incorporation in barley coleoptiles after incubation periods of 6 hr or longer) but it was consistent for all incubation periods. At 100 µg/ml, picloram slightly enhanced the incorporation of ^{14}C-ATP and ^{14}C-leucine by both hypocotyl and coleoptile sections in the first 6 hr but after that it inhibited such incorporation, particularly between 8 and 24 hr after beginning the incorporation. Picloram at 500 µg/ml, a growth-inhibiting concentration, drastically suppressed the incorporation of labeled precursors into RNA and protein of soybean hypocotyl and barley coleoptile tissue. The inhibition was not significant for the first 2 hr of incubation, but was highly significant thereafter. Evidently it takes some time before enough picloram has entered the tissue to exert an inhibitory effect on RNA and protein biosynthesis.

The experiments described included concentration and time series (Figures 9, 10, and 11) which made it possible to separate initial promoting and subsequent inhibiting effects which occur as picloram enters the tissue and is accumulated (?) at the sites of

RNA and protein synthesis. Thus picloram at 100 µg/ml takes about 6 hr to accumulate in the tissue in quantities sufficient to initiate an inhibitory effect on growth and on RNA and protein biosynthesis. After 8 hr enough picloram has entered the tissue to result in a serious inhibition of the processes studied. It should be emphasized then that in experiments with picloram or any other auxin-herbicide on excised plant tissues care must be taken in interpreting the results when the chemical is being used at only one concentration and for a single incubation period (e.g. picloram at 100 µg/ml for 6 hr). An example in the literature is the work of Moreland *et al.* (111) who did not find any effect of picloram on ^{14}C-ATP and ^{14}C-leucine incorporation into RNA and protein of soybean hypocotyl sections. It is possible that picloram, at the concentration (6×10^{-4} M, approximately 148 µg/ml) and incubation period (6 hr) used in their studies, initially promoted and subsequently inhibited RNA and protein biosynthesis but, without time-course experiments, the investigators were unable to detect such effects.

In excised barley leaf sections and Canada thistle leaf discs picloram at 10 µg/ml only slightly enhanced the incorporation of ^{14}C-labeled precursors into RNA and protein (Table 9). At 500 µg/ml, however, picloram drastically inhibited such incorporation. Inhibition of incorporation of ATP and leucine was slightly greater in Canada thistle leaf discs (82 and 69 per cent, respectively, relative to controls) than in barley leaf sections (72 and 59 per cent, respectively).

TABLE 9

Effect of picloram on RNA and protein biosynthesis, measured as incorporation of ^{14}C-labeled precursors, in excised barley leaf sections and Canada thistle leaf discs.[a]

Tissue	Picloram, µg/ml	RNA		Protein	
		10^{-3} x dpm[b]	%[c]	10^{-3} x dpm[b]	%[c]
Barley leaf sections	0	146.3		203.9	
	10	156.0	107	216.8	106
	500	41.0	28	90.0	44
Canada thistle leaf discs	0	79.2		168.1	
	10	90.3	114	178.8	106
	500	14.0	18	51.6	31

[a] Excised barley leaf sections (13 mm in length) and Canada thistle leaf discs (14 mm in diameter) were incubated for 8 hr in light (10220 lux) at 25°C in 0.01 M phosphate buffer (pH 6.0), containing 1 per cent sucrose, 20 µg/ml streptomycin sulfate, different concentrations of picloram, and 0.1 µCi/ml of ^{14}C-8-ATP or 0.05 µCi/ml of ^{14}C-U-leucine.

[b] Radioactivity in RNA or protein is represented as dpm per g initial fresh weight of tissue.

[c] Expressed as per cent of corresponding control.

From the results reported in the foregoing pages it is clear that an increase or decrease in RNA and protein contents of excised soybean hypocotyls, barley coleoptiles, barley leaf sections, and Canada thistle leaf discs by picloram (Table 8) is not totally dependent upon an increase or decrease in biosynthesis of new RNA and protein. For instance, an increase in incorporation of ^{14}C-ATP into RNA and of ^{14}C-leucine into protein brought about by picloram at 10 µg/ml did not result in an increase in RNA and protein content of barley coleoptile sections. On the other hand, there was an increase in RNA and protein contents of barley coleoptiles treated with picloram at 500 µg/ml, although incorporation of labeled precursors was drastically inhibited. Similar observations were made in soybean hypocotyl, barley leaf sections, and Canada thistle leaf discs. Picloram evidently strongly influences the RNA and protein levels in these excised tissues by affecting net loss of RNA and protein as well as the synthesis of new RNA and protein.

The magnitude of the response of excised plant tissues to picloram, especially at auxinic concentration (10 µg/ml), as regards growth and RNA and protein biosynthesis varied to a great extent with the tissue involved. Excised segments from elongating zones of etiolated soybean and barley seedlings were much more responsive than green leaf tissue from barley and Canada thistle. The implication is that the differences in the potential for cell elongation of different kinds of tissues are responsible for their differential response to picloram at growth-promoting concentration (10 µg/ml). At herbicidal concentration of picloram (500 µg/ml), however, this difference in response is not so

clear-cut.

It is important to mention here that picloram, depending upon concentration, promoted or inhibited the uptake of ^{14}C-8-ATP and ^{14}C-U-leucine by excised soybean hypocotyl and barley coleoptile sections (Table 10). An increase in uptake of ^{14}C-labeled ATP and leucine by picloram at 10 µg/ml may be, at least partially, responsible for an increase in their incorporation into RNA and protein (Figures 10 and 11). Similarly, an inhibition of uptake of labeled ATP and leucine by picloram at 500 µg/ml may be responsible for part of the inhibition of their incorporation. Since uptake of ^{14}C-labeled precursors is less affected than their incorporation into RNA or protein, a direct effect of picloram on net incorporation (after correcting for differences in uptake) accounts, at least in part, for an increase (at 10 µg/ml) or decrease (at 500 µg/ml) in RNA and protein biosynthesis by picloram. For instance, at 10 µg/ml picloram enhanced the uptake of ^{14}C-leucine by 24 per cent after 6 hr incubation (Table 10) while under the same conditions its incorporation into protein was increased by 38 per cent over that in controls (Figure 11). Differences between effects on uptake and incorporation of ^{14}C-labeled precursors were even more clear-cut at a growth-inhibiting concentration (500 µg/ml) of picloram. In barley coleoptiles, for example, uptake of ^{14}C-leucine after 6 hr incubation was inhibited by 58 per cent, whereas its incorporation into protein was inhibited by 83 per cent, relative to controls.

TABLE 10

Effect of picloram on uptake of ^{14}C-8-ATP and ^{14}C-U-leucine by excised soybean hypocotyl and barley coleoptile sections.[a]

Time, hr	Picloram, µg/ml	^{14}C-8-ATP				^{14}C-U-leucine			
		Barley coleoptile		Soybean hypocotyl		Barley coleoptile		Soybean hypocotyl	
		10^{-2} x dpm[b]	%[c]	10^{-2} x dpm[b]	%[c]	10^{-2} x dpm[b]	%[c]	10^{-2} x dpm[b]	%[c]
2	0	934		599		1053		744	
	10	1059	114	677	113	1142	109	979	132
	500	804	86	354	59	807	77	380	51
4	0	2230		1695		1876		1915	
	10	2588	116	1943	115	2188	117	2305	120
	500	1360	61	526	31	1094	58	600	31
6	0	3523		2399		3048		3348	
	10	4339	123	2728	114	3778	124	3628	108
	500	1617	46	715	30	1285	42	737	22
8	0	5093		3740		4187		4494	
	10	6493	128	4140	111	5055	121	4648	103
	500	2006	39	911	24	1439	34	1003	22

[a] Excised sections were incubated in the dark at 25°C in 5 ml of 0.01 M phosphate buffer (pH 6.0) containing 1 per cent sucrose, 20 µg/ml streptomycin sulfate, and different concentrations of picloram. ^{14}C-8-ATP and ^{14}C-U-leucine were added at 0.1 µCi and 0.05 µCi per ml of incubation medium, respectively.

[b] Radioactivity is represented as dpm/g initial fresh weight of tissue.

[c] Expressed as per cent of corresponding control.

III. Cytoplasmic Distribution of RNA and Protein Biosynthesis

The subcellular site of promoting or inhibiting effects of picloram on RNA and protein biosynthesis in soybean hypocotyl and barley coleoptile tissue was investigated. The results are presented in Tables 11 and 12. Incorporation of radioactivity from ^{14}C-labelled precursors into RNA and protein of particulate and supernatant fractions (separated at 20,000 x g) appeared to be equally sensitive to picloram at 10 µg/ml. For example, in soybean hypocotyl the radioactivity in particulate and soluble RNA and protein was increased by about 27 and 21 per cent and 10 and 9 per cent, respectively, over corresponding controls. The inhibition of ^{14}C-ATP and ^{14}C-leucine incorporation by picloram at 500 µg/ml, however, was greater in the soluble than in the particulate fraction (except in soybean hypocotyl where no difference in ^{14}C-leucine incorporation was observed). It is generally agreed (23, 66, 88) that most cellular RNA is synthesized within the nucleus (included in the particulate fraction in these experiments) and is transferred to the soluble fraction. Thus it is possible that any effect on biosynthesis of new RNA in the particulate fraction will also be observed in the soluble fraction. This was, in fact, the case in the experiments described. Further, it seems that picloram at 500 µg/ml inhibited the net transfer of newly synthesized RNA from the nucleus (particulate fraction) to the soluble fraction. Since protein synthesis is dependent on such RNA, the above effect in turn will also be reflected in greater inhibition of protein biosynthesis in the soluble fraction than in the particulate fraction (Table 11).

TABLE 11

Effect of picloram on cytoplasmic distribution of RNA biosynthesis, measured as incorporation of ^{14}C-8-ATP, in excised soybean hypocotyl and barley coleoptile tissue.[a]

Picloram, µg/ml	Soybean hypocotyl		Barley coleoptile	
	10^{-2} x dpm[b]	%[c]	10^{-2} x dpm[b]	%[c]
	Particulate Fraction			
0	43		82	
10	55	127	96	118
500	8	18	22	28
	Soluble Fraction			
0	697		856	
10	847	121	1017	119
500	60	9	151	18

[a] Excised soybean hypocotyl and barley coleoptile sections were incubated for 8 hr in the dark at 25°C in 0.01 M phosphate buffer (pH 6.0) containing 1 per cent sucrose, 20 µg/ml streptomycin sulfate, different concentrations of picloram, and 0.1 µCi/ml of ^{14}C-8-ATP. Particulate and soluble fractions were separated by centrifuging at 20000 x g for 30 min.

[b] Radioactivity in RNA is represented as dpm/g initial fresh weight.

[c] Expressed as per cent of corresponding control.

TABLE 12

Effect of picloram on cytoplasmic distribution of protein biosynthesis, measured as incorporation of ^{14}C-U-leucine in excised soybean hypocotyl and barley coleoptile tissue.[a]

Picloram, µg/ml	Soybean hypocotyl		Barley coleoptile	
	10^{-2} x dpm[b]	%[c]	10^{-2} x dpm[b]	%[c]
	Particulate Fraction			
0	364		465	
10	400	110	613	132
500	41	11	73	16
	Soluble Fraction			
0	940		933	
10	1022	109	1119	120
500	91	10	131	14

[a] Excised soybean hypocotyl and barley coleoptile sections were incubated in the dark at 25°C for 8 hr in 0.01 M phosphate buffer (pH 6.0) containing 1 per cent sucrose, 20 µg/ml streptomycin sulfate, different concentrations of picloram and 0.05 µCi/ml of ^{14}C-U-leucine. Particulate and soluble fractions were separated by centrifuging at 20000 x g for 30 min.

[b] Radioactivity in protein is represented as dpm/g initial fresh weight.

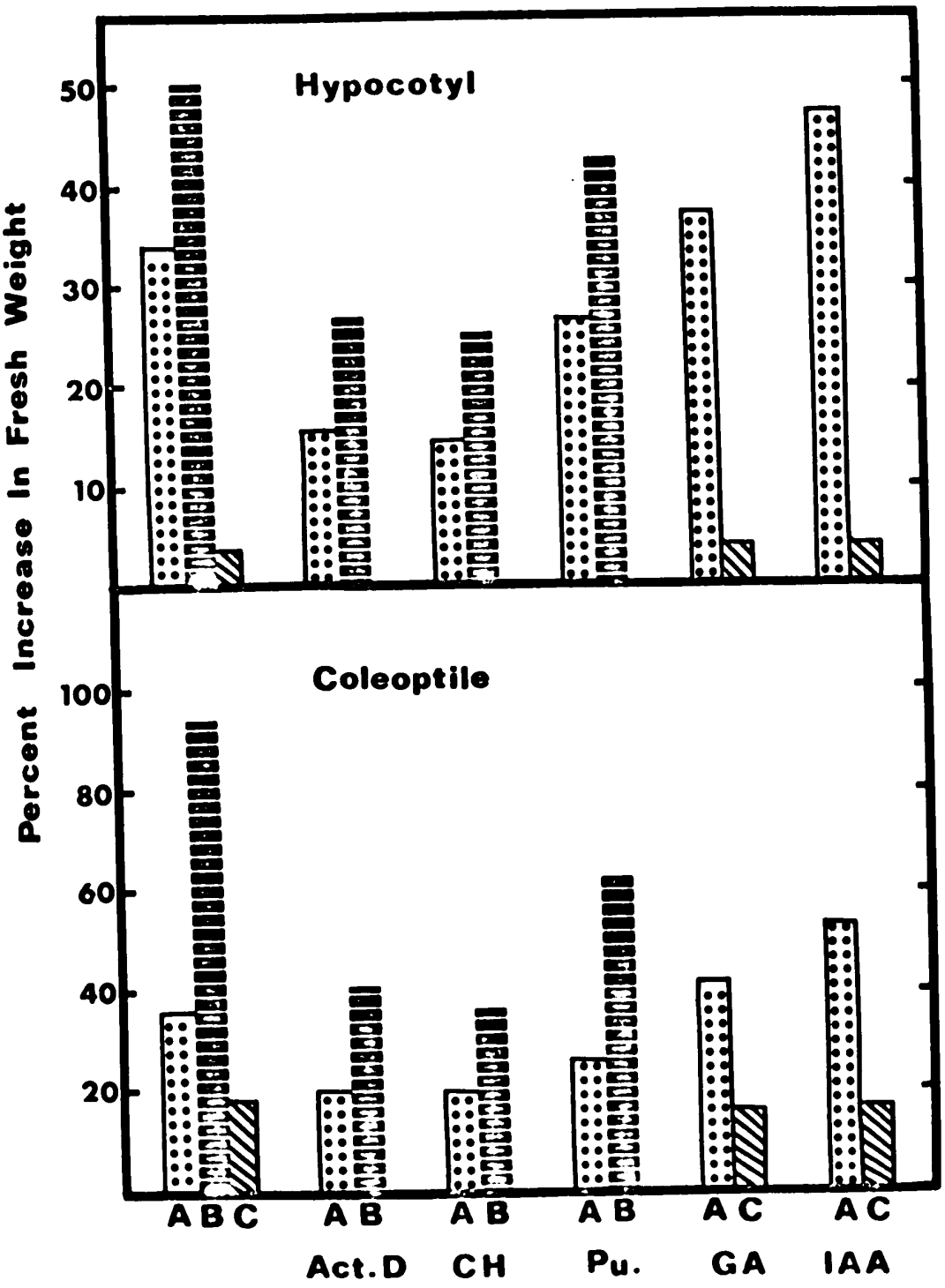
[c] Expressed as per cent of corresponding control.

IV. Growth and RNA and Protein Biosynthesis in the Presence of Inhibitors and Other Growth Regulators

Actinomycin D, cycloheximide, and puromycin were tested for their capacity to alter the normal and picloram-induced growth and RNA and protein biosynthesis of excised soybean hypocotyl and barley coleoptile tissue. Gibberellic acid and indoleacetic acid were also used for comparison with picloram. The results of these experiments are illustrated in Figures 12, 13, and 14.

Actinomycin D and cycloheximide in the incubation medium inhibited both the normal (control) and picloram-induced growth in soybean hypocotyl and barley coleoptile sections during an 8-hr incubation period (Figure 12). Picloram-induced growth was inhibited more than control growth. For example, in barley coleoptiles picloram-induced and control growth were inhibited by 60 and 45 per cent, respectively. Puromycin at the concentration used in this study (40 µg/ml, approximately 9.0×10^{-5} M) was not very effective in inhibiting the growth of soybean hypocotyl and barley coleoptile sections. It inhibited growth by only 30 per cent. Picloram (10 µg/ml), GA, and IAA all enhanced growth, although they did so at different concentrations and to varying degrees. Picloram as an auxin, however, appeared to be several times more effective in enhancing growth than either GA or IAA. When picloram at 500 µg/ml was used together with GA or IAA, the inhibition of growth was equal or somewhat greater than with picloram alone. A similar additive effect has been reported for picloram and IAA on the growth of excised oat coleoptile sections by Schrank (140).

Figure 12. Effect of picloram (A, B, and C), actinomycin D (Act. D), cycloheximide (CH), puromycin (Pu), GA, and IAA on growth, as per cent increase in fresh weight, of excised soybean hypocotyl (upper) and barley coleoptile (lower) sections. The sections were incubated for 8 hr in the dark at 25°C in 5 ml of 0.01 M phosphate buffer (pH 6.0) containing 1 per cent sucrose, 20 µg/ml streptomycin sulfate, and 0(A), 10(B), or 500(C) µg/ml picloram. Concentrations of actinomycin D, cycloheximide, puromycin, GA, and IAA were 10, 2, 40, 50, and 50 µg/ml. respectively. Data plotted are means of three experiments.


Hypocotyl
Coleoptile
Percent Increase In Fresh Weight
50
40
30
20
10
100
80
60
40
20
A B C
A B
A B
A B
A C
A C
Act.D
CH
Pu.
GA
IAA

Both actinomycin D and cycloheximide drastically inhibited the uptake and incorporation of ^{14}C-ATP and ^{14}C-leucine by soybean hypocotyl and barley coleoptile sections (Figures 13 and 14). The incorporation of labeled precursors into RNA and protein was inhibited much more than their uptake by the tissue. Picloram at growth-promoting concentration enhanced RNA and protein biosynthesis (precursor incorporation) and this picloram-induced synthesis also was greatly inhibited by actinomycin D and cycloheximide. Actinomycin D, for example, inhibited the control and picloram-induced RNA biosynthesis by about 76 and 88 per cent, respectively, in soybean hypocotyl and barley coleoptile sections. The two inhibitors were equally effective in suppressing RNA and protein biosynthesis, except that in some cases actinomycin D was more effective than cycloheximide on RNA biosynthesis. Cycloheximide inhibited RNA and protein synthesis by about 50 and 80 per cent, respectively, in soybean hypocotyl and barley coleoptile sections. My results on inhibition of RNA and protein synthesis by actinomycin D and cycloheximide are in agreement with those of Click and Hackett (39) on potato tuber discs and of Key *et al.* (82, 84) on soybean hypocotyl who used actinomycin D and cycloheximide at concentrations similar to those used in the present experiments. As in growth, puromycin also was not very effective in inhibiting protein biosynthesis (Figure 14). Inhibition of control and picloram-induced growth (Figure 12) of soybean hypocotyl and barley coleoptile sections by actinomycin D, cycloheximide, and puromycin paralleled the inhibition of RNA and protein biosynthesis (Figures 13 and 14). GA and IAA elicited the same general enhancement of RNA and protein biosynthesis

Figure 13. Effect of picloram (A, B, and C), actinomycin D (Act. D), cycloheximide (CH), GA, and IAA on uptake of ^{14}C-8-ATP (total area of the bars) and its incorporation into RNA (shaded area of the bars) of excised soybean hypocotyl (upper) and barley coleoptile (lower) sections. The sections were incubated for 8 hr in the dark at 25°C in 0.01 M phosphate buffer (pH 6.0) containing 1 per cent sucrose, 20 µg/ml streptomycin sulfate, 0(A), 10(B), or 500(C) µg/ml picloram, and 0.1 µCi/ml of ^{14}C-8-ATP. Concentrations of actinomycin D, cycloheximide, GA, and IAA were 10, 2, 50, and 50 µg/ml respectively. Radioactivity in RNA is represented as dpm/g initial fresh weight. Data plotted are means of three experiments.

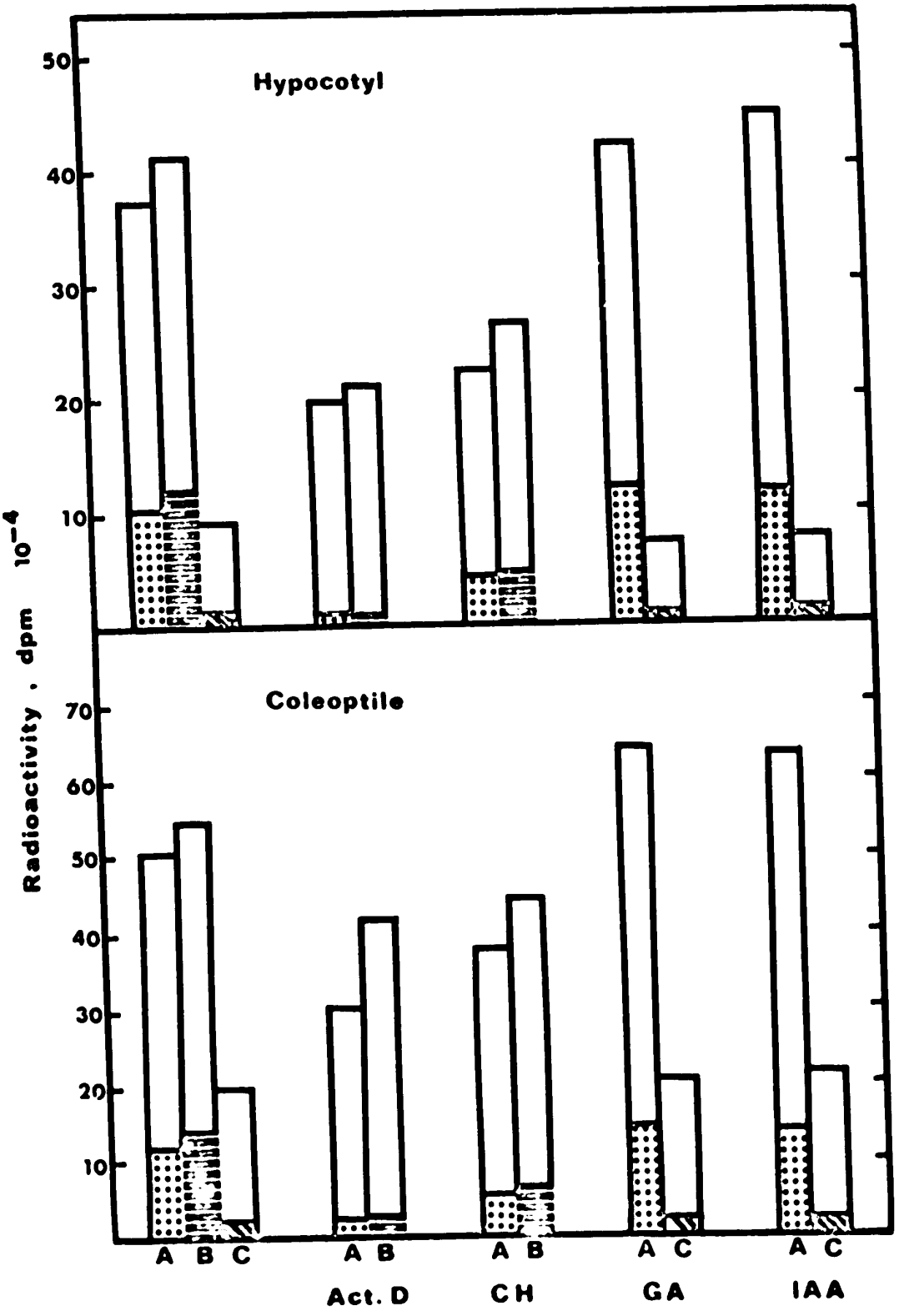
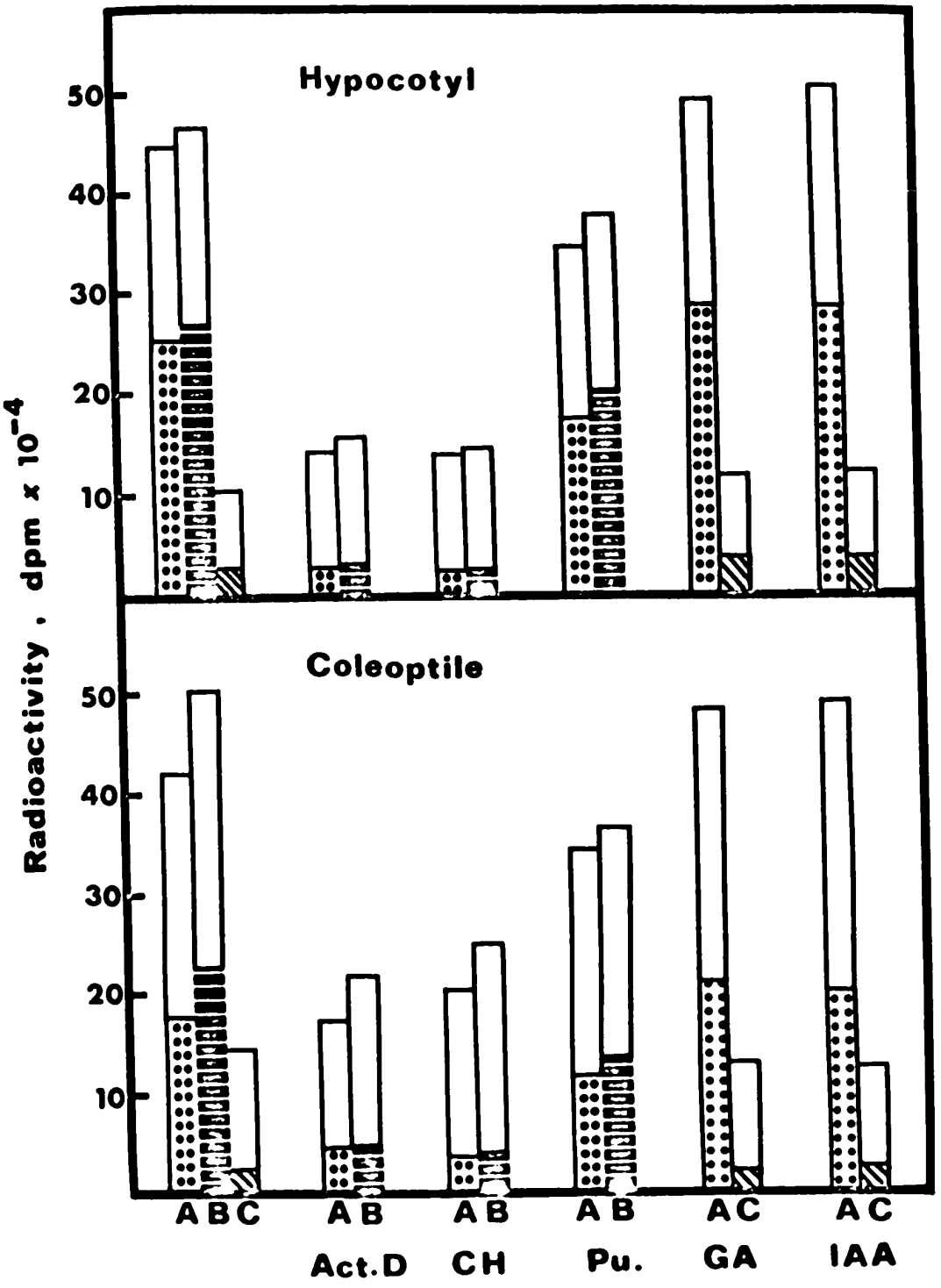

Hypocotyl
Coleoptile
Radioactivity . dpm 10⁻⁴
50
40
30
20
10
70
60
50
40
30
20
10
A B C
A B
A B
A C
A C
Act. D
CH
GA
IAA

Figure 14. Effect of picloram (A, B, and C), actinomycin D (Act. D), cycloheximide (CH), puromycin (Pu), GA, and IAA on uptake of ^{14}C-U-leucine (total area of the bars) and its incorporation into protein (shaded area of the bars) of excised soybean hypocotyl (upper) and barley coleoptile (lower) sections. The sections were incubated for 8 hr in the dark at 25°C in 5 ml of 0.01 M phosphate buffer (pH 6.0) containing 1 per cent sucrose, 20 µg/ml streptomycin sulfate, 0(A), 10(B), or 500(C) µg/ml picloram, and 0.05 µCi/ml ^{14}C-U-leucine. Concentrations of actinomycin D, cycloheximide, puromycin, GA, and IAA were 10, 2, 40, 50, and 50 µg/ml, respectively. Radioactivity in protein is represented as dpm/g initial fresh weight. Data plotted are means of three experiments.


Hypocotyl
Coleoptile
Radioactivity . dpm x 10^{-4}
50
40
30
20
10
A B C
A B
Act.D
A B
CH
A B
Pu.
A C
GA
A C
IAA

as did picloram, although they did so at different concentrations and to varying degrees. Promotion of growth and RNA and protein biosynthesis by GA and IAA, at concentrations similar to those used in this study, in various excised plant tissues, is now well established (20, 28, 43, 65, 82, 118). In the experiments reported here, picloram was considerably more effective than either GA or IAA in enhancing growth and RNA and protein biosynthesis at a lower concentration. In similar experiments on growth (cell elongation) of a number of excised plant tissues, Kefford and Caso (79) observed that picloram was either equal to or more effective than IAA in promoting growth when both were used at similar concentrations.

As discussed earlier (p. 79), changes in uptake of ^{14}C-labeled precursors are responsible, at least in part, for the promotion or inhibition of their incorporation into RNA and protein by various compounds tested. The experiments reported below, were designed to eliminate any direct effects of exogenous picloram and other compounds on uptake of labeled precursor.

Soybean hypocotyl and barley coleoptile sections were subjected to various pretreatments with actinomycin D, cycloheximide, picloram (both at a growth-promoting and a growth-inhibiting concentration), GA, and IAA. Such pretreated tissues were then incubated for 3 hr in a buffered solution of ^{14}C-leucine.

Pretreatment of soybean hypocotyl and barley coleoptile sections for a total of 6 hr in an auxinic concentration of picloram increased growth as well as incorporation of ^{14}C-leucine into protein

over that in control (buffer pretreatment) sections (Table 13). The increase in growth (about 50 per cent in soybean hypocotyl and 100 per cent in barley coleoptile) was much greater, however, than the increase in ^{14}C-leucine incorporation (8 to 14 per cent). Pretreatment of excised sections for 6 hr in either actinomycin D or cycloheximide considerably reduced both growth and ^{14}C-leucine incorporation. Treatment with these inhibitors for 3 hr after 3 hr pretreatment with picloram effectively inhibited picloram-induced growth and ^{14}C-leucine incorporation. On the other hand, picloram relieved, to some extent, the inhibiting effects of prior treatment with actinomycin D and cycloheximide on growth and on incorporation of ^{14}C-leucine. The level of growth and ^{14}C-leucine incorporation did not reach that of controls (6 hr buffer pretreatment) in any of these promotor-inhibitor treatment sequences. The first 3 hr pretreatment always had a greater effect on growth and on ^{14}C-leucine incorporation than the later 3 hr. For example, if inhibitor pretreatment was given in the first 3 hr, growth and ^{14}C-leucine incorporation were inhibited more than if it was given in the second 3 hr. These observations indicate that the inhibitors and picloram probably do not affect the pre-existing 'growth essential protein' and 'RNA' but influence their synthesis, and that it takes them more than 3 hr to completely do so. The inhibitor experiments strongly suggest that growth of excised soybean hypocotyl and barley coleoptile sections depends upon continued RNA and protein synthesis. The picloram-induced component of growth of excised tissues is dependent upon additional (new) RNA and additional protein biosynthesis.

TABLE 13

Effect of various pretreatment sequences of picloram, actinomycin D, and cycloheximide on growth and on ^{14}C-leucine incorporation into protein of excised soybean hypocotyl and barley coleoptile sections.[a]

Pretreatment (3 hr, 3 hr)	Soybean hypocotyl		Barley coleoptile	
	Fresh weight[b]	10^{-2} x dpm[c]	Fresh weight[b]	10^{-2} x dpm[c]
Buffer, Buffer	26[d]	644	36	579
Pic.$_{10}$, Pic.$_{10}$	40	699	69	668
Act. D, Act. D	14	262	22	268
CH, CH	14	245	19	256
Act. D, Pic.$_{10}$	18	369	38	354
CH, Pic.$_{10}$	20	368	40	355
Pic.$_{10}$, Act. D	25	448	45	445
Pic.$_{10}$, CH	25	432	41	464

a Excised soybean hypocotyl and barley coleoptile sections were pretreated for a total of 6 hr (3 hr, 3 hr) in 5 ml of 0.01 M phosphate buffer (pH 6.0) containing 1 per cent sucrose, 20 µg/ml streptomycin sulfate, without or with picloram (10 µg/ml), actinomycin D (10 µg/ml), or cycloheximide (CH, 2 µg/ml). All sections were then incubated for 3 hr in buffer containing 0.1 µCi/ml ^{14}C-U-leucine. All incubations were carried out in the dark at 25°C.

b Expressed as per cent increase over the initial fresh weight.

c Radioactivity incorporated into protein is represented as dpm per g initial fresh weight of tissue.

d Each figure in the table is the mean of three experiments.

Pretreatment of soybean hypocotyl and barley coleoptile sections with GA or IAA (both at 50 µg/ml) enhanced growth and ^{14}C-leucine incorporation though the increase was less than that following picloram at 10 µg/ml (Table 14). Pretreatment of sections for 6 hr in picloram at 500 µg/ml, on the other hand, inhibited growth and subsequent ^{14}C-leucine incorporation. More inhibition occurred when picloram was supplied in the first 3 hr of the pretreatment period than if it was supplied in the second 3 hr. Although total exposure time to exogenous picloram was the same in both instances, it is clear that in the former instance picloram had more time to act within the tissue and as a result exerted a greater inhibitory effect.

The effects of supplying promoting and inhibiting substances separately, in sequence, indicate that sufficient amounts of the various compounds have to penetrate and accumulate in the tissue, probably at the site(s) of their action, before growth and ^{14}C-leucine incorporation (or other processes) show a detectable response. This suggests that their action is not likely to be at the cell surface and lies with some constituent, probably RNA and/or protein, in the cell.

F. Uptake and Cellular Distribution of ^{14}C-Picloram by Excised Plant Tissues

I. Uptake

Excised soybean hypocotyl and barley coleoptile sections continued to absorb picloram for at least 24 hr from buffer solutions containing 0.05 µCi/ml (approximately 11.8 µg/ml) ^{14}C-picloram

TABLE 14

Effect of various pretreatment sequences of picloram, GA, and IAA on growth and on ^{14}C-leucine incorporation into protein of excised soybean hypocotyl and barley coleoptile sections.[a]

Pretreatment (3 hr, 3 hr)	Soybean hypocotyl		Barley coleoptile	
	Fresh weight[b]	10^{-2} x dpm[c]	Fresh weight[b]	10^{-2} x dpm[c]
Buffer, Buffer	26	644	36	579
$Pic._{500}$, $Pic._{500}$	11	196	21	194
GA, GA	29	697	40	667
IAA, IAA	33	682	51	618
GA, $Pic._{500}$	20	455	33	387
$Pic._{500}$, GA	14	338	27	288
IAA, $Pic._{500}$	22	460	31	293
$Pic._{500}$, IAA	15	348	27	247

a Excised soybean hypocotyl and barley coleoptile sections were pretreated for 6 hr (3 hr, 3 hr) in 0.01 M phosphate buffer (pH 6.0) containing 1 per cent sucrose, 20 µg/ml streptomycin sulfate, without or with picloram (500 µg/ml), GA (50 µg/ml), or IAA (50 µg/ml). All sections were then incubated for 3 hr in buffer containing 0.1 µCi/ml ^{14}C-leucine. Incubations were carried out in the dark at 25°C.

b Expressed as per cent increase over initial fresh weight.

c Radioactivity from ^{14}C-leucine incorporated into protein is represented as dpm per g initial fresh weight of tissue.

d Each figure in the table is the mean of three experiments. Figures for control (buffer, buffer) are the same as reported in Table 13.

(Figure 15). Except for a rapid initial entry of picloram the uptake was almost linear for at least the first 8 hr. Soybean hypocotyl sections absorbed more picloram than barley coleoptile sections. Since the uptake of growth regulators has been shown to occur mainly through the cut surface of excised segments (161, 164), it is reasonable to assume that the larger cut surface area of soybean hypocotyl sections may, at least partly, account for a greater absorption of picloram by them as compared to coleoptile sections of the same length.

During an 8 hr incubation period each barley leaf section (13 mm in length) and Canada thistle leaf disc (14 mm in diameter) absorbed about 2500 and 3500 dpm, respectively, from buffer solutions containing 0.05 μCi/ml ^{14}C-picloram. During this period barley leaf sections accumulated ^{14}C-picloram to a concentration about 1.5 times as high (170,000 dpm/g fresh weight) as that of the external medium (112,000 dpm/ml).

Loss of radioactivity from excised soybean hypocotyl, barley coleoptile, barley leaf sections, and Canada thistle leaf discs was observed when an 8-hr treatment with 0.05 μCi/ml of ^{14}C-picloram in 0.01 M phosphate buffer (pH 6.0) was followed by leakage periods of 2 and 4 hr in 'clean' 0.01 M phosphate buffer (pH 6.0) (Table 15). The leakage phenomenon varied in rate and extent in the different tissues. Loss of radioactivity was greatest in the first 2 hr. During the 4-hr period about 30 per cent of the total radioactivity in the tissue was released into the ambient buffer from ^{14}C-picloram-treated soybean hypocotyl and barley coleoptile sections. Leaf sections released only

Figure 15. Time-course of uptake of ^{14}C-picloram by excised soybean hypocotyl and barley coleoptile sections. The radioactivity is represented as dpm x 10^{-2} per hypocotyl or coleoptile section. Excised sections were incubated in the dark at 25°C in 5 ml of 0.01 M phosphate buffer (pH 6.0) containing 0.05 µCi/ml (approximately 11.8 µg/ml) ^{14}C-picloram. Data plotted are means of three experiments.

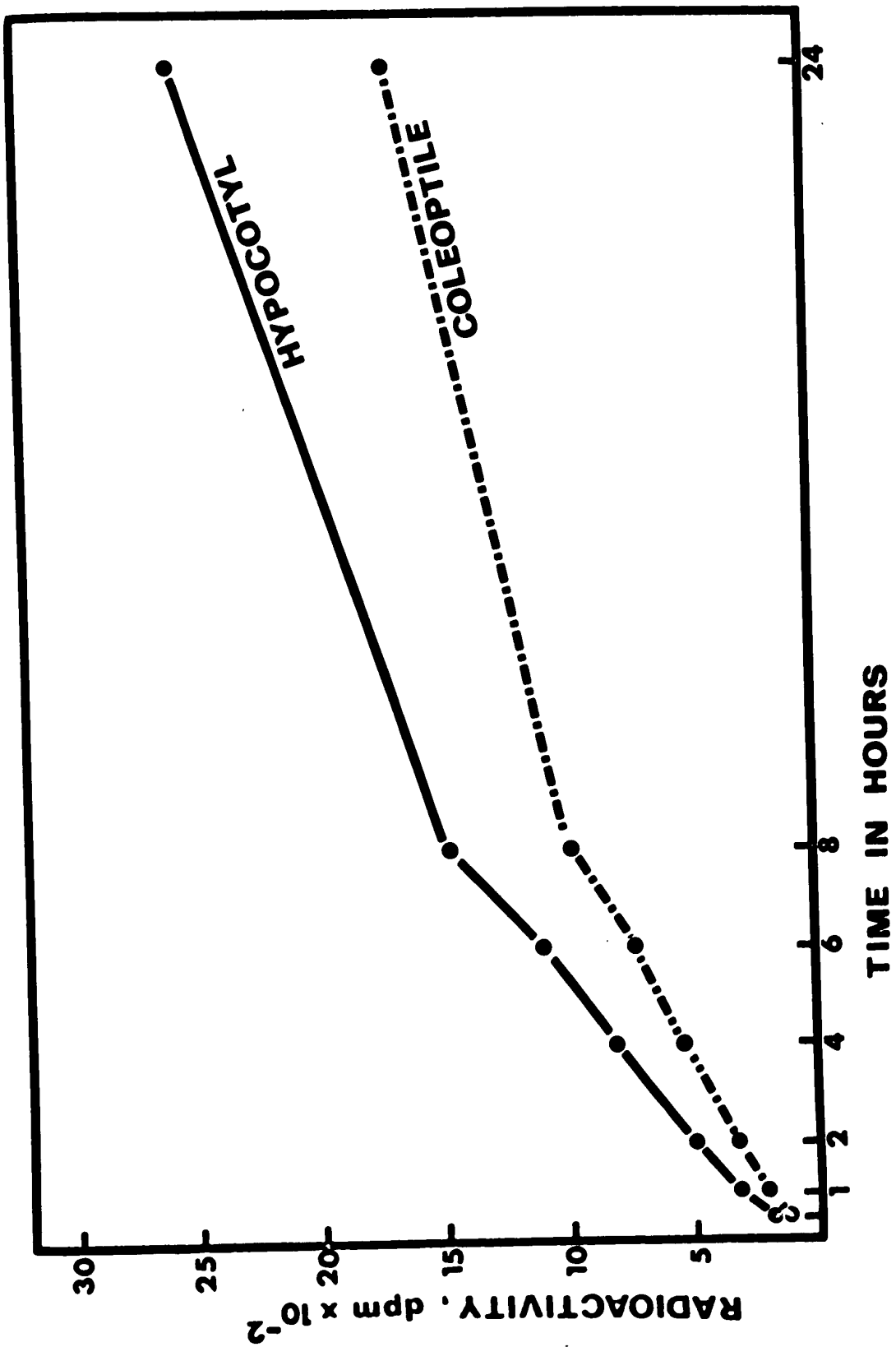

HYPOCOTYL
COLEOPTILE
RADIOACTIVITY, dpm x 10⁻²
TIME IN HOURS
30
25
20
15
10
5
1
2
4
6
8
24

TABLE 15

Loss of radioactivity from ^{14}C-picloram treated excised soybean hypocotyls, barley coleoptiles, barley leaf sections, and Canada thistle leaf discs incubated for 2 and 4 hr in 0.01 M phosphate buffer (pH 6.0) at 25°C.[a]

Tissue	Radioactivity in ambient buffer[b]	
	2 hr	4 hr
Soybean hypocotyl	21.9	30.5
Barley coleoptile	21.2	28.9
Barley leaf	13.0	18.3
Canada thistle leaf	10.4	15.8

[a] All excised tissues were treated with ^{14}C-picloram (0.05 µCi/ml, 11.8 µg/ml) in 0.01 M phosphate buffer (pH 6.0) for 8 hr at 25°C. Soybean hypocotyl and barley coleoptile sections were incubated in the dark and barley and Canada thistle leaf tissues in light (10220 lux).

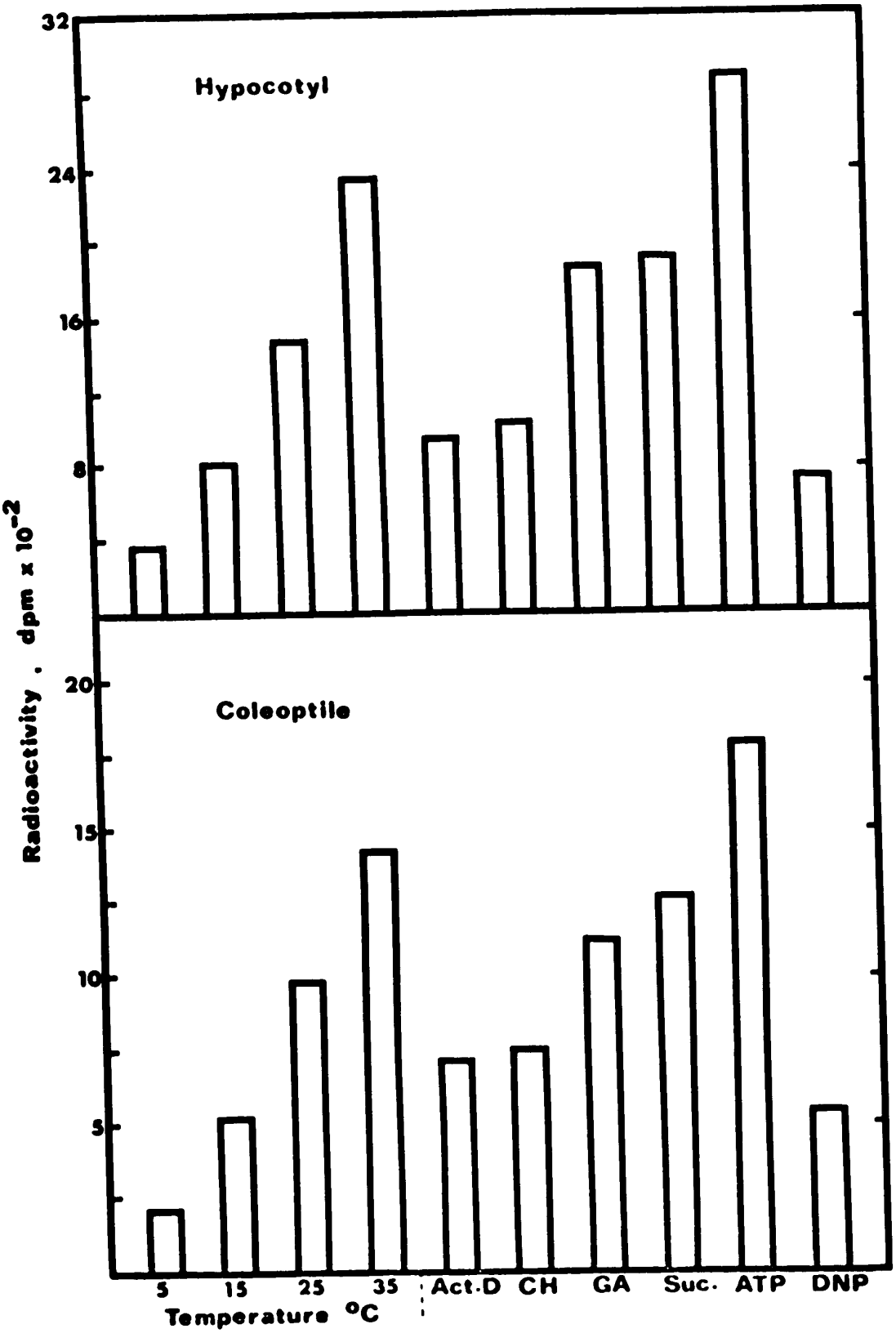
[b] Expressed as per cent of total absorbed.

16 to 18 per cent. A lower release of radioactivity from barley and Canada thistle leaf sections than from soybean hypocotyl and barley coleoptile sections could be attributed to a strong binding of ^{14}C-picloram by the leaf sections.

The results of experiments on the influence of temperature, actinomycin D, cycloheximide, GA, sucrose, ATP, and DNP on uptake of ^{14}C-picloram by soybean hypocotyl and barley coleoptile sections during an 8-hr incubation period are illustrated in Figure 16. An increase in temperature markedly increased the uptake of picloram both by soybean hypocotyl and barley coleoptile sections. The Q_{10} values were 2.2, 1.8, and 1.6 for soybean hypocotyl and 2.5, 1.9, 1.6 for barley coleoptile sections in the temperature range from 5° to 35°C. Obviously, in both tissues, an increase in temperature in the lower range (5° to 15°C) brought about a greater increase in uptake than a similar increase in temperature in the upper range (25° to 35°C). The Q_{10} values fall between values normally encountered for purely passive (Q_{10} 1.0 to 1.3) and active (Q_{10} 2.0 to 3.0) processes. The observations suggest, therefore, that picloram uptake is made up of both passive and active processes.

Actinomycin D and cycloheximide, at concentrations which inhibited RNA and protein biosynthesis in soybean hypocotyl and barley coleoptile sections (Figures 13 and 14) resulted in an inhibition of uptake of ^{14}C-picloram by these tissues (Figure 16). The two inhibitors reduced uptake by 35 and 30 per cent in soybean hypocotyls and by 28 and 24 per cent in barley coleoptiles, respectively.

Figure 16. Effect of temperature (5, 15, 25, and 35°C), actinomycin D (Act. D, 10 µg/ml), cycloheximide (CH, 2 µg/ml), GA (50 µg/ml), sucrose (suc., 2 per cent), ATP (4×10^{-3} M), and DNP (1×10^{-4} M) on uptake of ^{14}C-picloram by excised soybean hypocotyl (upper) and barley coleoptile (lower) sections. Sections were routinely incubated for 8 hr in the dark at 25°C in 5 ml of 0.01 M phosphate buffer (pH 6.0) containing 0.05 µCi/ml ^{14}C-picloram. The radioactivity is represented as dpm $\times 10^{-2}$ per hypocotyl or coleoptile section. Data plotted are means of three experiments.


Hypocotyl
Coleoptile
Radioactivity . dpm x 10^{-2}
32
24
16
8
20
15
10
5
5
15
25
35
Act.D
CH
GA
Suc.
ATP
DNP
Temperature °C

GA enhanced uptake by 14 and 26 per cent in soybean hypocotyl and barley coleoptile sections, respectively (Figure 16). The interaction between plant growth regulators during their uptake by excised plant tissues has interested a number of investigators (37, 74, 75) and the uptake of one growth regulator has been shown to be affected by another.

If the uptake of picloram is assumed to be an 'active', energy-requiring process, then it follows that the addition of energy sources such as sucrose and ATP, or the addition of DNP, a metabolic inhibitor, to the incubation medium should influence the uptake. The addition of sucrose (2 per cent) or ATP (4×10^{-3} M) greatly increased the uptake of ^{14}C-picloram by soybean hypocotyl and barley coleoptile sections (Figure 16). Sucrose increased uptake by about 27 per cent, whereas ATP almost doubled it. DNP, on the other hand, reduced the uptake by about half. These results strongly suggest that an energy relationship is involved in the uptake of picloram. Similar suggestions have been made earlier by other workers for uptake of picloram (16, 73) and other growth regulators (37, 75, 161).

II. Subcellular Distribution

The distribution of radioactivity in various subcellular fractions, separated by differential centrifugation of aqueous extracts of ^{14}C-picloram-treated excised tissues, was determined (Table 16). More than 95 per cent of the activity was in the soluble fraction. In barley and Canada thistle leaf tissue about 2 per cent of the total absorbed radioactivity was found in the chloroplast-rich fraction. Although care

TABLE 16

Subcellular distribution of ^{14}C-picloram, represented as per cent of total absorbed, in excised soybean hypocotyls, barley coleoptiles, barley leaf sections, and Canada thistle leaf discs.[a]

Subcellular Fraction[b]	Soybean hypocotyl	Barley coleoptile	Barley leaf	Canada thistle leaf
Nuclei-rich	2.2	2.2	1.0	1.0
Chloroplast-rich	-	-	2.4	1.9
Mitochondria-rich	1.2	1.5	1.5	1.6
Soluble	96.6	96.3	95.1	95.5

[a] All excised sections were incubated for 8 hr at 25°C in 0.01 M phosphate buffer (pH 6.0) containing 0.05 µCi/ml (11.8 µg/ml) ^{14}C-picloram. Soybean hypocotyl and barley coleoptile sections were incubated in the dark and barley and Canada thistle leaf tissues in light (10220 lux).

[b] Subcellular fractions: 0 - 4500 x g for 10 min for soybean hypocotyl and barley coleoptile tissue or 0 - 200 x g for 5 min for leaf tissue (nuclei-rich); 200 - 4500 x g for 10 min for leaf tissue (chloroplast-rich); 4500 - 20,000 x g for 30 min (mitochondria-rich); 20,000 x g supernantant (soluble).

was taken to avoid contamination of subcellular fractions by washing and recentrifuging them twice, the possibility that nuclei, chloroplast, and mitochondrial fractions are contaminated with each other cannot be ruled out. The cellular distribution of picloram in these tissues is similar to that of 2,4-D in the experiments of Hallam and Sargent (62) who found most of the radioactivity in the soluble fraction of aqueous extracts of ^{14}C-2-4-D-treated bean leaves. However, in non-aqueous extracts of bean leaf, chloroplasts were the principal cell components to contain radioactivity from 2,4-D. From my results it seems that either picloram did not enter into the nuclei, chloroplast, and/or mitochondria or some of the activity from these organelles leaked out or was actively excreted into the supernatant during aqueous extraction of the tissue. The latter possibility has been confirmed by Hallam and Sargent (62) for 2,4-D and its metabolites in bean leaves.

G. Translocation of ^{14}C-Picloram in Canada Thistle, Soybean, and Barley

Plant mounts and autoradiograms in Figure 17 show the distribution of radioactivity in Canada thistle, soybean, and barley plants 1 day after application of ^{14}C-picloram on a single leaf. The radioactive material in the plants was chiefly in the form of unaltered picloram as determined by chromatographic analysis of the plant extracts (see Metabolism, Section H.II). Therefore, the movement of the label in the plants represented mainly translocation of the applied herbicide.

Picloram moved out of the treated leaf and was translocated both downward and upward in the leaves and stem of all three species.

Figure 17. Distribution of radioactivity in Canada thistle (A), soybean (B), and barley (C), one day after application of 0.1 μCi of ^{14}C-picloram (0.2 μCi in Canada thistle) to a single leaf as indicated by the arrows. The lettered photographs represent the plants, the corresponding photographs to the right of them the autoradiograms.


A
B
C

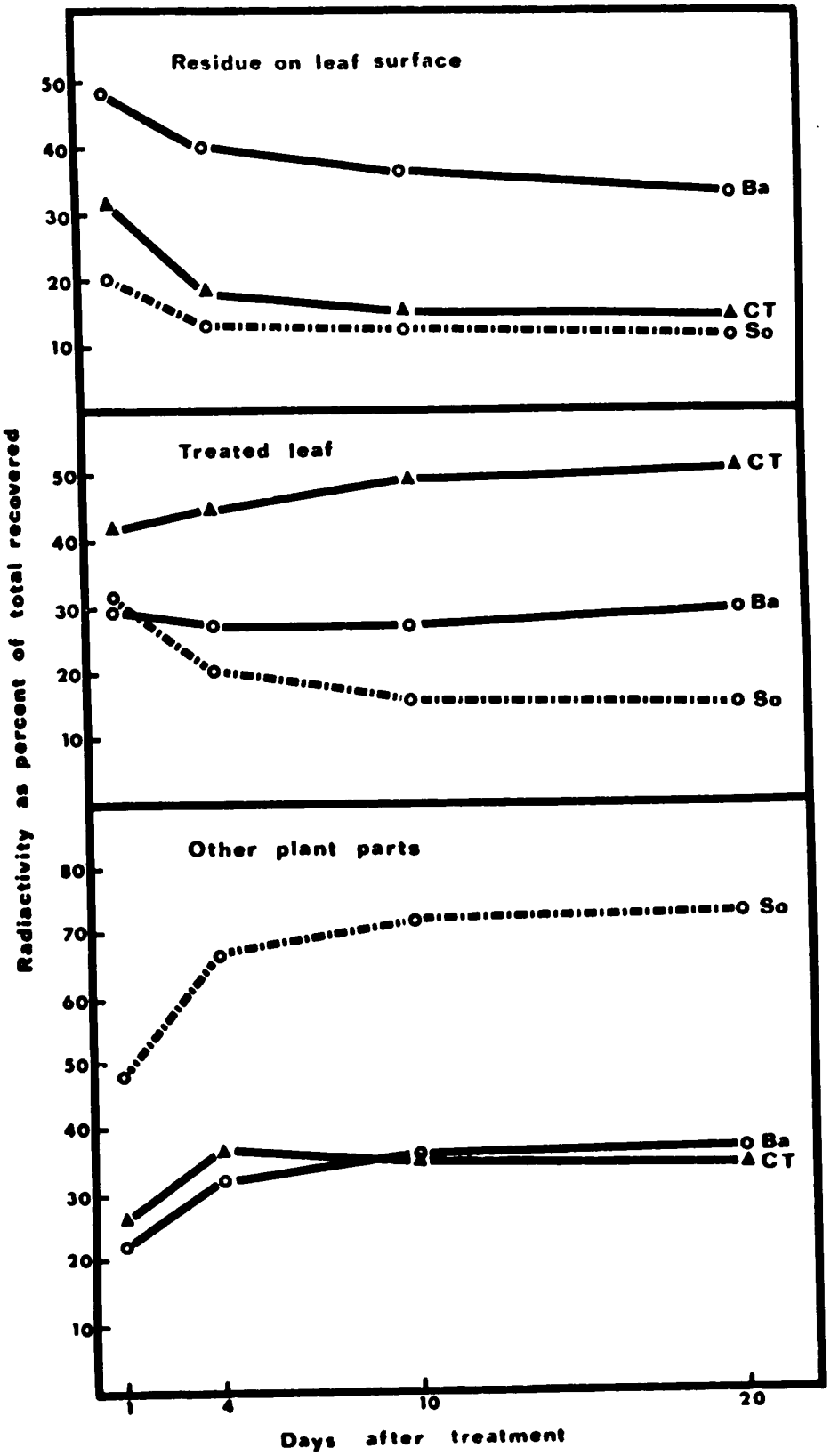

A
B
C

The ^{14}C label accumulated in actively developing young leaves at the shoot apex of Canada thistle and soybean plants, whereas in barley plants it was more or less evenly distributed throughout the green tissue. More of the total radioactivity appeared to remain in the treated leaf of Canada thistle and barley than in soybean plants.

Quantitative data on the absorption and translocation of ^{14}C-picloram in the three species after foliar application were obtained at time intervals ranging from one to twenty days (Figure 18). Entry of picloram into the leaves of Canada thistle and soybean was much faster and more complete than into barley leaves, as judged from the amount of picloram residue left on the leaf surface. About 80 - 85 per cent of the total radioactivity recovered four or more days after treatment had entered the leaves of Canada thistle and soybean as compared to only 60 per cent in barley. The amount of radioactivity translocated out of the treated leaf to other parts of the plant, one or more days after treatment, was greatest for soybean and about the same for Canada thistle and barley. About 50 per cent of the total recovered radioactivity in Canada thistle, 30 per cent in barley, and 15 per cent in soybean, was retained by the treated leaf 20 days after treatment. The results are in agreement with those of autoradiographic studies.

Total recovery of radioactivity from plants 20 days after treatment with ^{14}C-picloram, as a fraction of the dose applied, was 55 per cent in barley, 64 per cent in Canada thistle, and 75 per cent in soybean. Radioactivity exuded by roots (146), evolved as $^{14}CO_2$ (see Metabolism, Section H.II), or incorporated in the ethanol-insoluble

Figure 18. Time-course of ^{14}C-picloram absorption and translocation in Canada thistle (CT), soybean (So), and barley (Ba), after application of 0.1 µCi of ^{14}C-picloram to a single leaf. Radioactivity is presented as a percentage of the total amount recovered. Data plotted are means of two replications of four plants each.


Residue on leaf surface
Ba
CT
So
Treated leaf
CT
Ba
So
Other plant parts
So
Ba
CT
Radioactivity as percent of total recovered
Days after treatment

residue, accounted for less than 3 per cent of the dose. The decrease in recovery with time probably is attributable to volatilization of picloram (59) despite the fact that volatility losses from a glass slide or parafilm in 24 hr were negligible (147). The distribution patterns of radioactivity after root uptake of ^{14}C-picloram (Figure 19) were similar to those following leaf application, except that translocation was more extensive and that there was more activity in the roots after absorption via the nutrient solution. The label was more or less evenly distributed throughout the plants of Canada thistle, with slight accumulation of radioactivity in the young leaves. In soybeans most of the activity was in the main axis, that is, the young trifoliate leaves, stem, and roots, and very little activity appeared in the two primary leaves.

H. Metabolism of ^{14}C-Picloram

I. Excised Plant Tissues

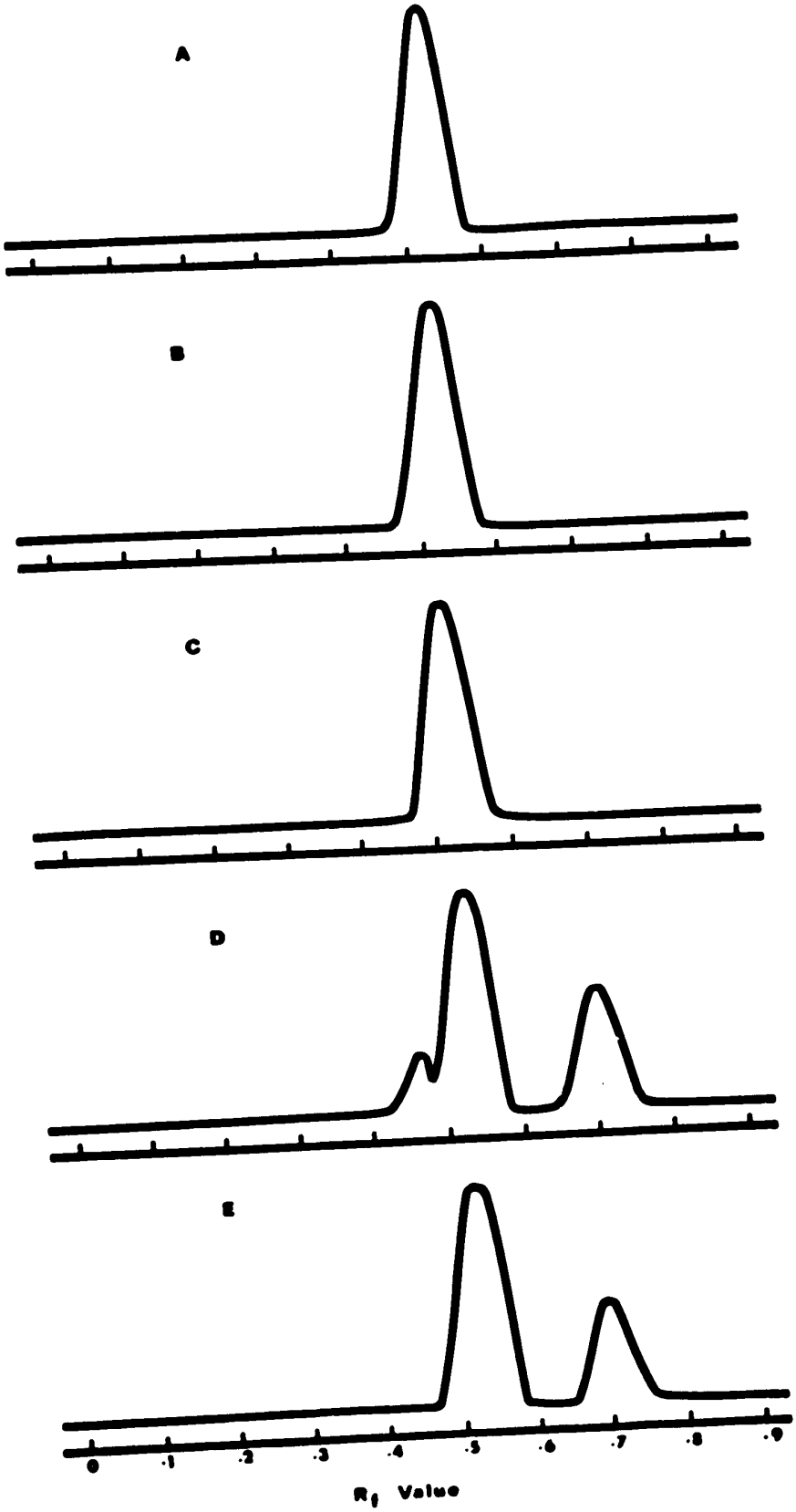
Scans of paper chromatograms showed radioactivity from ^{14}C-picloram in the treatment solution as a single peak at a mean Rf 0.53 (Figure 20,A). After 1-day treatment periods all the radioactivity in ethanol extracts of all the excised plant tissues was at the picloram position. After 3-day treatment periods radioactive material in the extracts of soybean hypocotyl (Figure 20,B) and barley coleoptile (Figure 20,C) also ran as single peaks at the picloram position. In barley and Canada thistle leaf extracts (Figure 20,D and E, respectively) an additional peak appeared at Rf 0.70 and in barley leaf extracts a

Figure 19. Distribution of radioactivity in Canada thistle (A), soybean (B), and barley (C), one day after root uptake of ^{14}C-picloram from nutrient solution (0.4 µCi in 100 ml). The lettered photographs represent the plants, the corresponding photographs to the right of them the autoradiograms.


A
C


A
B
C

Figure 20. Distribution of radioactivity along paper chromatograms of ^{14}C-picloram (A), and ethanol extracts of sections of soybean hypocotyl (B), barley coleoptile (C), barley leaf (D), and Canada thistle leaf (E), three days after treatment with 0.05 µCi/ml of ^{14}C-picloram in 0.01 M phosphate buffer (pH 6.0). Chromatographic solvent: n-butanol : ammonium hydroxide (28 per cent) : water, 8:1:1.

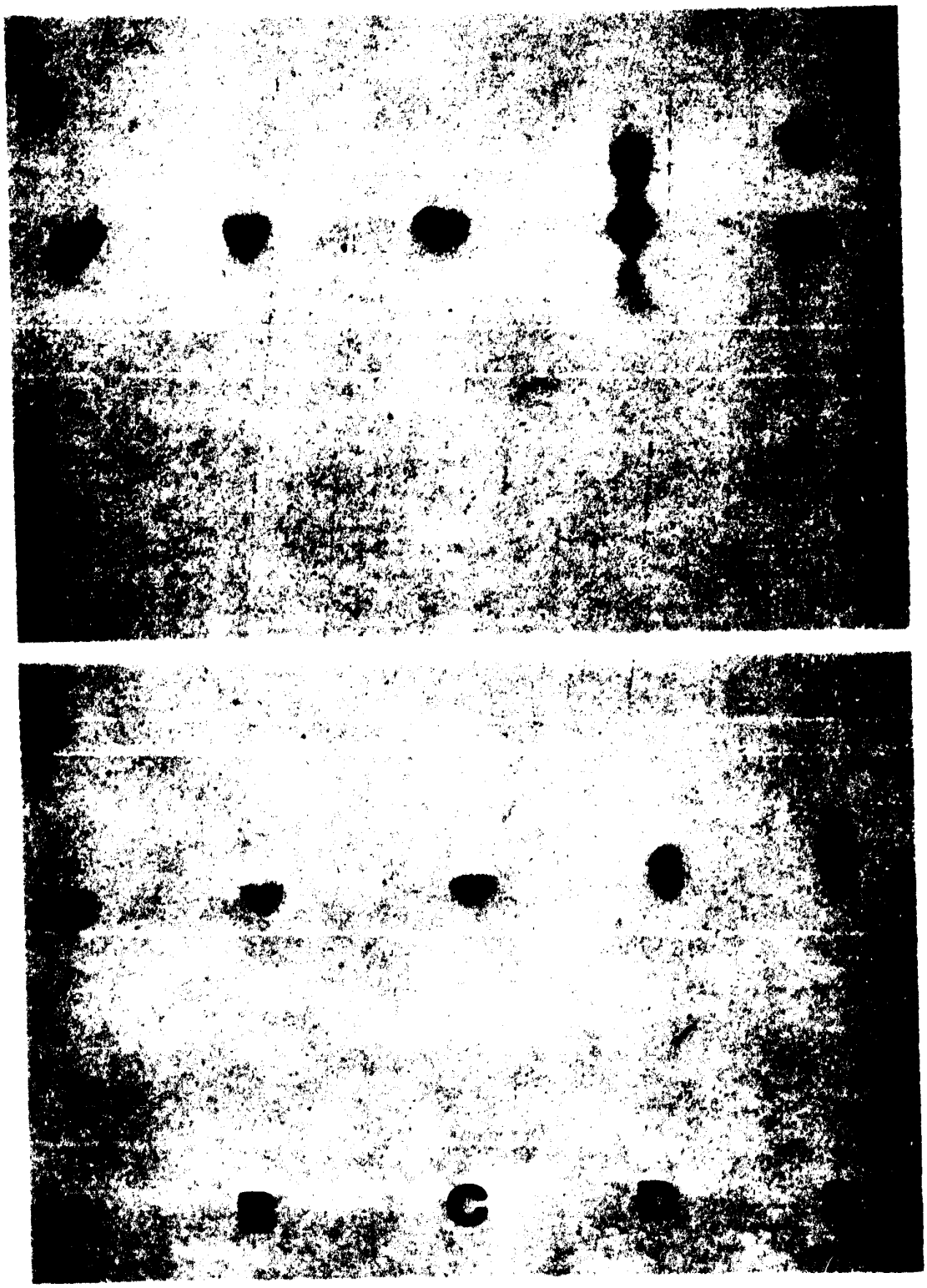

A
B
C
D
E
0
.1
.2
.3
.4
.5
.6
.7
.8
.9
R_f Value

small amount of activity was present at Rf 0.48. Thin-layer chromatography of ethanol extracts confirmed the paper chromatographic results (Figure 21, upper). The radioactivity present at positions other than that of unaltered picloram accounted for about 40 and 25 per cent of the total activity in barley and Canada thistle leaf tissue, respectively.

It has been reported (107, 128) that picloram or its metabolites may form conjugates with plant constituents. Thus, the additional peaks or spots might represent metabolites of picloram or conjugated products of picloram or its metabolites. If they were conjugates, acid or alkaline hydrolysis should release the compounds from the plant constituent moiety. Therefore, the tissue extracts were hydrolyzed with acid and then chromatographed again. Thin-layer chromatography of the hydrolyzed extracts (Figure 21, lower) showed that the spots representing ^{14}C-picloram remained at the original position and the additional spots from barley and Canada thistle leaf extracts had disappeared. All the radioactivity in the hydrolyzed extracts thus appeared at the picloram position. These results suggest that picloram was conjugated with plant constituents in the excised barley and Canada thistle leaf sections and that it was released from the conjugation by acid hydrolysis. On chromatograms, the conjugates gave a positive reaction for sugar with benzidine and a negative reaction for amino acid with ninhydrin. It appears, therefore, that some picloram is conjugated with sugars (s) in excised leaf tissues.

In another experiment, in barley and Canada thistle leaf tissues, less than 1 per cent of the total absorbed radioactivity was

Figure 21. Autoradiograms of thin-layer chromatograms of ^{14}C-picloram (A), and ethanol extracts (upper) and hydrolyzed extracts (lower) of sections of soybean hypocotyl (B), barley coleoptile (C), barley leaf (D), and Canada thistle leaf (E), three days after incubation in 0.01 M phosphate buffer (pH 6.0), containing 0.05 µCi/ml of ^{14}C-picloram. Thin-layer of 0.25 mm silica gel GF on glass plates was developed in n-butanol : ethanol : water, 2:2:1 (v/v/v).



bound to the macromolecules (ethanol-, TCA-, and phenol-insoluble material) three days after treatment with ^{14}C-picloram.

II. Intact Plants

a. Decarboxylation

Canada thistle, soybean, and barley plants were treated with 0.1 µCi of ^{14}C-picloram on a single leaf. Figure 22 shows cumulative total radioactivity collected as $^{14}CO_2$ from plants which received a total amount of labeled herbicide equivalent to 220,000 dpm per plant. It is evident that some decarboxylation of ^{14}C-picloram occurred in all three species, but the rate and extent were extremely low. During a period of 20 days, the total radioactivity collected was less than 0.05 per cent of the dose applied.

b. Metabolites in Plant Extracts

Chromatographic analysis of ethanol extracts of plants foliarly treated with ^{14}C-picloram showed that metabolism of this herbicide did not occur in any of the plant species tested. Autoradiograms of thin-layer chromatograms of ethanol extracts from Canada thistle, soybean, and barley, 10 and 20 days after treatment with ^{14}C-picloram (Figure 23, upper and lower, respectively) showed no labeled compounds other than unaltered picloram. In Canada thistle plants even 40 days after treatment no evidence of metabolism of ^{14}C-picloram was obtained and only unaltered chemical was recovered from the ethanol extracts of treated plants.

Figure 22. Radioactivity collected as $^{14}CO_2$ (cumulative total) from Canada thistle, soybean, and barley plants which were treated with ^{14}C-picloram equivalent to 220,000 dpm per plant on a single leaf. Radioactivity is represented as dpm per plant. Data plotted are means of two replicates of two (Canada thistle), or four (soybean and barley) plants each.

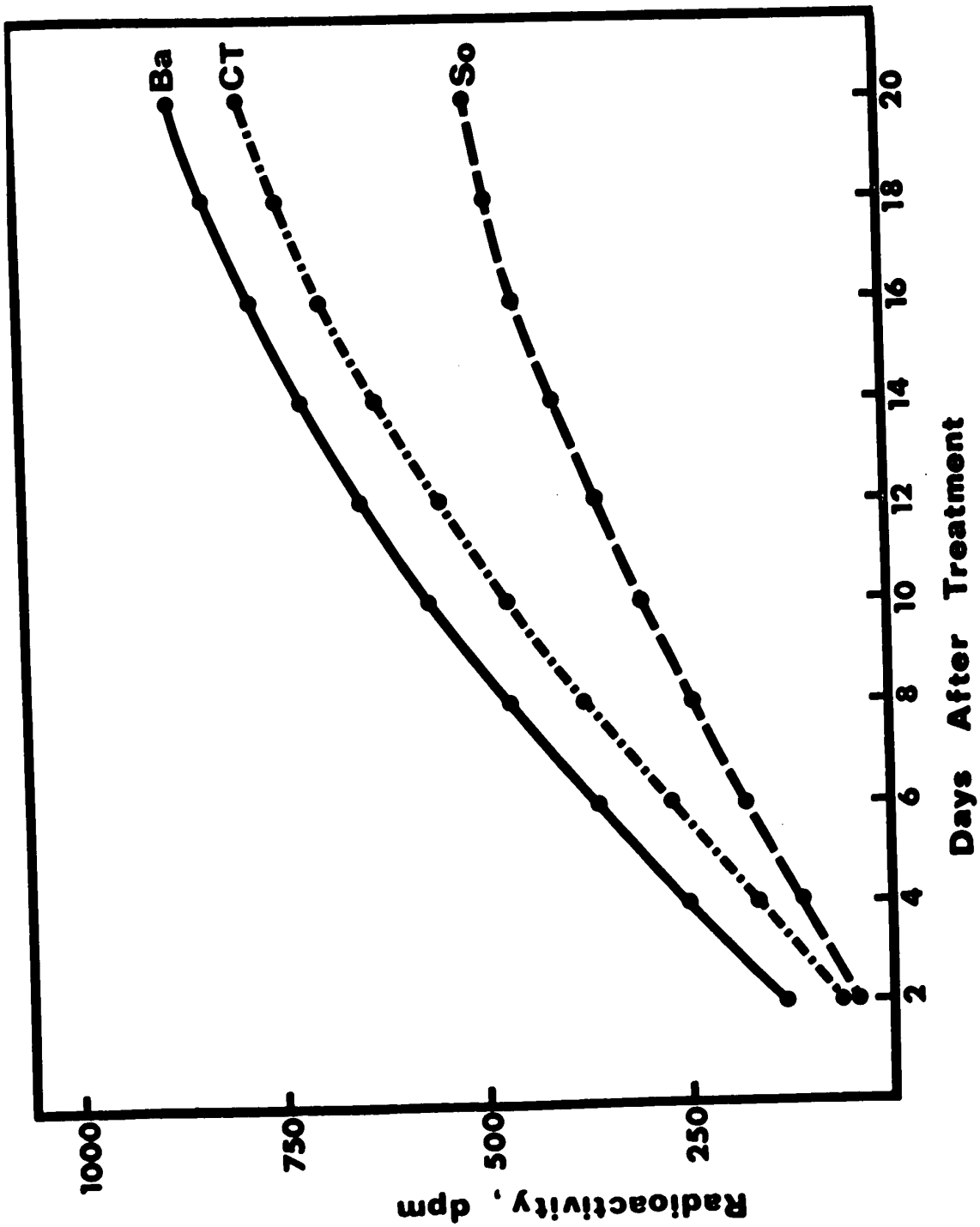
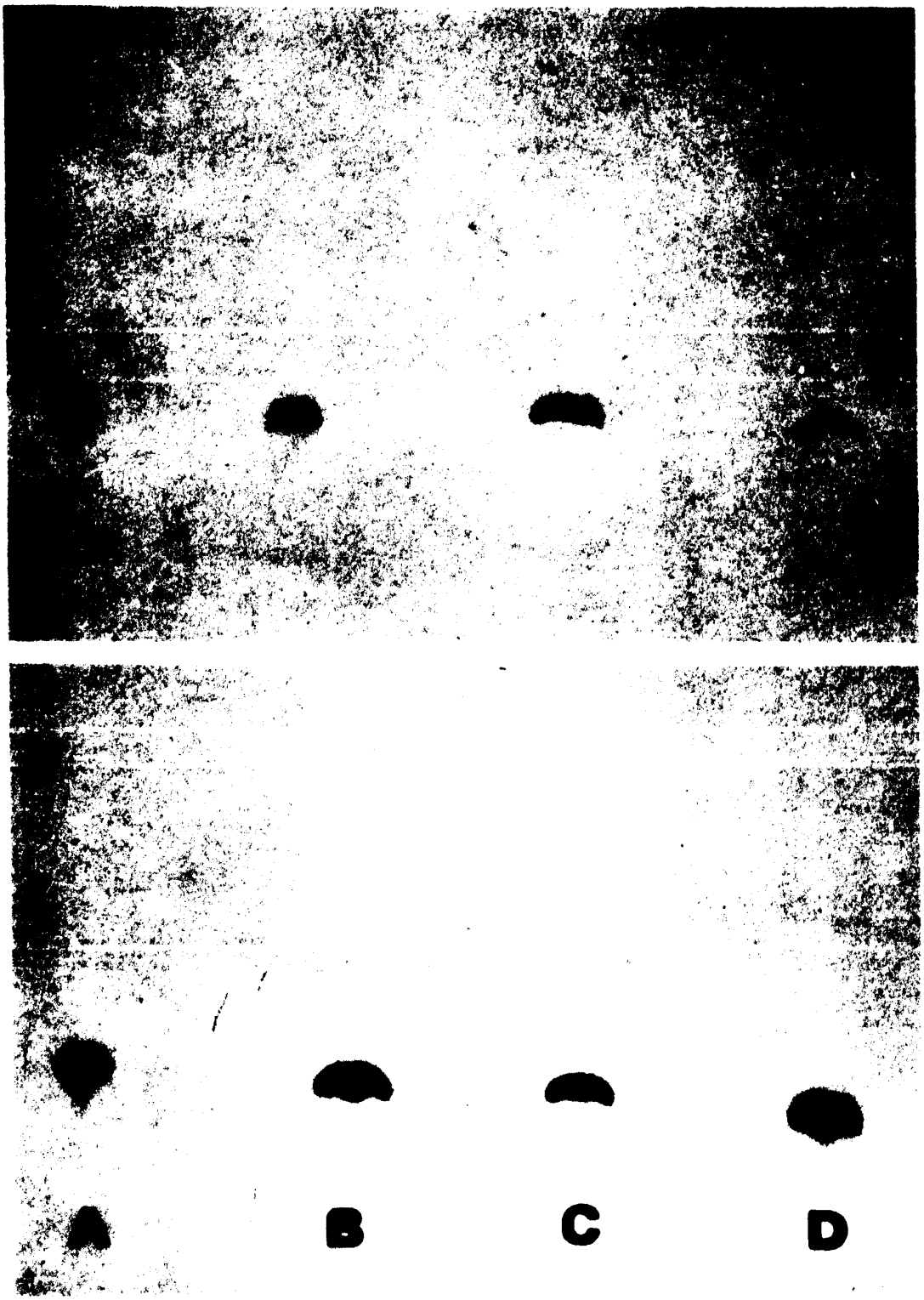

Ba
CT
So
1000
750
500
250
Radioactivity, dpm
2
4
6
8
10
12
14
16
18
20
Days After Treatment

Figure 23. Autoradiograms of thin-layer chromatograms of ^{14}C-picloram (A) and ethanol extracts of Canada thistle (B), soybean (C), and barley (D) plants, 10 days (upper) and 20 days (lower) after foliar application of 0.1 µCi of ^{14}C-picloram. Thin-layer of 0.25 mm silica gel GF on glass plate was developed in n-butanol : ammonium hydroxide (28 per cent) : water, 8:1:1 (v/v/v).


B
C
D

DISCUSSION AND CONCLUSIONS

The significance of certain observations related to each of the experiments described has been discussed in the appropriate sections. However, the implications of these experimental findings deserve more detailed attention.

A. $^{14}CO_2$-Fixation and ^{14}C-Assimilate Translocation

Photosynthetic $^{14}CO_2$-fixation in Canada thistle and soybean was reduced to about one-half of that in controls by 10 µg/ml (0.036 mM) picloram. This effective concentration level is similar to that of many potent inhibitors of photosynthetic $^{14}CO_2$-fixation such as phenylureas and s-triazines (8, 9, 40) which, at 0.05 mM or less, reduced fixation in bean and soybean to less than 5 per cent of that in controls. On the other hand, the 10 mM concentration of picloram required for 50 per cent inhibition of the Hill reaction in the experiments of Hamill and Switzer (64) was far greater than the concentration of phenylureas or s-triazines needed to achieve the same effect (110). These observations suggest that the inhibitory effect of picloram on photosynthesis is indirect and is a result of disturbances of other physiological processes. A reduction in chlorophyll concentration in picloram-treated plants because of destruction of chlorophyll or an interference with its formation may be, at least partially, responsible for the observed inhibition of photosynthetic $^{14}CO_2$-fixation in picloram-sensitive soybeans and Canada thistle. The reduced chlorophyll content, by lowering

the photosynthetic efficiency of the chloroplasts, could account for a reduction in dry weight of picloram-treated plants, an explanation which has been provided for 2,4-D action (174).

Young soybean plants are much more sensitive to picloram than Canada thistle plants, but primary leaves of soybean, the main photosynthesizing organs at the growth stage examined, did not show greater inhibition of $^{14}CO_2$-fixation by picloram than did Canada thistle leaves. This observation can be explained in part by the results of translocation studies on picloram in these species. Only very small amounts of radioactivity accumulated in the primary leaves of soybean plants 1 day after root treatment with ^{14}C-labeled picloram (Figure 19). Most of the picloram had accumulated in young trifoliolate leaves. In Canada thistle, on the other hand, picloram was distributed throughout all leaves with only limited accumulation in young leaves, after similar treatment. Differences in the distribution patterns of picloram after root uptake probably contribute more to the expression of selectivity than any other direct effects of the herbicide on photosynthetic processes. Nevertheless, such direct or indirect effects undoubtedly play an important role in the phytotoxicity of picloram.

If the interference of certain herbicides with photosynthesis consists of somehow incapacitating the photosynthetic units (quantasomes) in which the Hill reaction occurs (40, 109, 121), then $^{14}CO_2$-fixation would be reduced only in proportion to the number of units so inactivated, and the distribution of ^{14}C into various compounds should not be changed drastically. The significant changes in distribution of assimilated ^{14}C in various compounds brought about by picloram in these experiments,

therefore, again point to the indirect nature of the picloram effect on photosynthesis. The observed effects by themselves can hardly be considered disastrous to the plant but again, they undoubtedly are a part of the expression of picloram's phytotoxicity.

Increases in labeled malic acid, aspartic acid, glutamic acid, asparagine, and serine, as a result of picloram treatment suggest that alternate pathways for the synthesis of these compounds exist apart from the photosynthetic fixation pathway described by Calvin and coworkers (14). Three possibilities, linking the Embden-Meyerhof pathway and the Krebs cycle, and involving the formation of oxalacetic acid or malic acid from either pyruvic acid or phosphoenol pyruvic acid and CO_2, are evident in intermediary metabolism pathways (23). These intermediates (malic and oxalacetic acid) in turn, through known biochemical pathways (23) could give rise to increased amounts of the above-mentioned compounds in picloram-treated plants. These results are fully in agreement with those of Young (177) who observed an increase in asparagine, aspartic acid, and glutamic acid in picloram-treated field bindweed (susceptible to picloram), and a decrease in these compounds in Kochia (*Kochia scoparia* (L.) Schred.) (resistant). The increase in radioactivity in some amino acids after $^{14}CO_2$-fixation in Canada thistle and soybean in my experiments could account for the increase in protein content observed in these species after picloram treatment (Table 7).

Sucrose is the main constituent of assimilates entering the phloem in photosynthesizing leaves (68, 114, 125, 152, 167) and in the

present experiments a large part of the assimilated ^{14}C in Canada thistle, soybean, and corn was incorporated into sucrose (Table 8). If picloram directly inhibits sucrose transport, a back-up of (unlabeled) sucrose in the phloem might shift the incorporation of assimilated ^{14}C in the leaves away from sucrose to other compounds such as amino acids. No direct effects of picloram on phloem transport have been described in literature reports, however, possibly because of the difficulty of separating direct and indirect effects experimentally. A more probable explanation of the observed shift is that picloram caused a disturbance of growth in young leaves of Canada thistle and soybean, and that the consequent reduction in sink-activity caused a back-up of sucrose in the transport system. In corn, which is relatively resistant to picloram, no significant inhibition of assimilate transport and no significant shift in ^{14}C-distribution occurred. One would have to assume, of course, that leaf excision in Canada thistle and soybean just before exposure to $^{14}CO_2$ did not alter the sucrose gradient in the transport system sufficiently to affect ^{14}C-incorporation.

From the views put forth in the above paragraph it follows that the inhibition of translocation of ^{14}C-assimilates to young leaves in Canada thistle (Figure 6, B_{10} and C_{10}) and soybean (Figure 7, B_1 and C_1) also is an indirect rather than a direct result of picloram treatment.

The behavior of cotyledonary leaves of soybean in $^{14}CO_2$-fixation and ^{14}C-translocation may have some physiological significance. These cotyledons, as in several other species (see reference 29 in toto), were able to fix $^{14}CO_2$ in the light. Since the assimilation of carbon

dioxide in light by cotyledonary leaves appears to follow pathways which are operative in other photosynthesizing tissues (29) the process has considerable physiological significance with respect to the synthesis of important cellular constituents required for seedling growth and development. In the present experiments the translocation of ^{14}C fixed by cotyledonary leaves to young growing parts in soybean plants was reduced sharply by picloram. The retention of photosynthates in the cotyledonary leaves of picloram-treated soybean plants may be responsible for the retardation of senescence and abscission of such cotyledons observed in plants sprayed with picloram at levels which did not cause death of plants (p.51). Similar observations have been made recently by Fletcher *et al.* (54) on retardation of bean leaf senescence by benzyladenine.

My findings on inhibition of transport of ^{14}C-assimilates in Canada thistle and soybean contrast with those of Leonard *et al.* (97), who reported increased transport and accumulation of ^{14}C in stem tissues and petioles of picloram-treated grape, and those of Cardenas *et al.* (30) who found increased accumulation of assimilated ^{14}C in 2,4-D-treated cocklebur. Differences in plant species and their physiological conditions probably account for these differences. The findings described agree, however, with results of experiments on red kidney bean similar to those reported here (163).

My observations on inhibition of transport of ^{14}C-assimilates to roots correspond with the observations of Leonard *et al.* (96, 97) after the application of herbicidal dosages of picloram to several

woody species, but their suggestion of direct injury to phloem by picloram (96) was largely conjectural. A slow-developing detrimental effect of 2,4-D on translocation of amitrole in beans was reported by Crafts and Yamaguchi (42). They suggested that the failure of amitrole to be translocated was due to loss of the ability of the sieve tubes to hold amitrole by increasing the permeability of sieve tube membranes. Such reasoning could explain the failure of ^{14}C-assimilates to be translocated when herbicidal doses of picloram are used. Destruction of the phloem of stem and root in picloram-treated plants has been reported (52, 61, 80) and may contribute to the inhibition of assimilate translocation in picloram-treated soybean and Canada thistle plants in the present experiments. The blocking of assimilate transport to roots in picloram-treated Canada thistle and soybean probably is related also to decreased production of ^{14}C-sucrose and reduced sink-activity in the roots. Since the visible response of roots to picloram is much less spectacular than that of young leaves, the effect of the former on assimilate export from leaves probably is less important than the effect of the latter, as expressed in decreased ^{14}C-sucrose production.

The results of these experiments illustrate differential responses of sensitive and resistant species to picloram treatment, as expressed in production and translocation of labeled assimilates after exposure to $^{14}CO_2$. Except for a strong indication that these changes are brought about largely indirectly, after growth disturbances in young growing leaf tissue and also perhaps root tissue caused by disturbances of other physiological processes, they do not elucidate the question of primary point of attack of the herbicide. Nevertheless, such indirect

effects undoubtedly play an important role in the phytotoxicity of picloram.

B. RNA and Protein Metabolism

Results of my experiments on RNA and protein contents of picloram-treated soybean, Canada thistle, and barley plants correspond with those reported in the literature for picloram (17, 64, 100) and other auxin-herbicides (12, 30, 36, 85, 173). The differences in RNA and protein levels of picloram-treated and -untreated cucumber and soybean plants in the experiments of Malhotra and Hanson (100) were small, suggesting that the concentration of herbicide may have been excessive. In my experiments differences in RNA and protein concentrations of susceptible soybean and Canada thistle and resistant barley plants and in excised plant tissues after picloram treatment (Tables 6, 7, and 8) may be explained on the basis of differences in degradative metabolism of RNA and protein, i.e., different levels of ribonuclease and proteinase, between the two groups of plants and differences in their capacity to synthesize new RNA and protein.

Picloram-treated soybean and Canada thistle plants showed, in addition to the inhibition of apical growth, a distinct swelling of the stem below the growing point. This swelling may be related to an overproduction of rRNA and free ribosomes, according to an explanation suggested as part of the mechanism of action of auxin-herbicides (66), and based on the accumulation of RNA in the form of free ribosomes in tissues treated with 2,4-D or picloram (36, 53, 83, 87, 100).

Changes in growth and RNA and protein metabolism of excised soybean hypocotyl and barley coleoptile sections after incubation can be schematically represented as follows:

Picloram, µg/ml	Growth	Total RNA or protein/section		
		Net change	Synthesis	Degradation
	Soybean hypocotyl			
0	+	--	++	----
10	++	+	+++	--
500	0	-	0	-
	Barley coleoptile			
0	+	---	++	-----
10	+++	--	+++	-----
500	↓	-	↓	-

In soybean hypocotyl a net increase in RNA and protein content relative to control and initial levels in response to picloram at growth-promoting concentration, was a result of increased synthesis of new RNA and protein and a decrease in RNA and protein loss during incubation. However, at a growth-inhibiting concentration of picloram, an increase in RNA and protein content over the controls (although a decrease relative to the initial level) was due to a large decrease in loss of RNA and protein during incubation. In barley coleoptile sections, an increase in RNA and protein content in response to picloram at a growth-promoting concentration was brought about mainly through an increase in new RNA and protein biosynthesis. Since there was no net increase in RNA and protein content in picloram-treated (10 µg/ml) barley coleoptiles, in spite of an increase in synthesis of new RNA and protein over the controls, it is reasonable to assume that the loss of RNA and protein in such

picloram-treated sections also was greater than in control sections. As in soybean hypocotyls, picloram at a growth-inhibiting concentration caused an increase in RNA and protein content over that in controls mainly through a decrease in net loss of RNA and protein.

The great increase in RNA and protein content of intact susceptible soybean and Canada thistle plants and of excised soybean hypocotyls after picloram treatment in these experiments thus is considered associated both with lower levels of ribonuclease (66, 100) and proteinase and an increase in the synthesis of new RNA and protein. The inability of intact plants and excised coleoptile sections of the resistant species barley to accumulate more RNA and protein after picloram treatment, in spite of an increase in the synthesis of new RNA and protein, is attributable to high levels of ribonuclease (66, 100) and proteinase in the tissue. Furthermore, picloram also may influence the activity of these degradative enzymes differently in susceptible and resistant species (7, 66, 100) and thus affect their RNA and protein levels differently.

In experiments on excised soybean hypocotyl and barley coleoptile sections, actinomycin D, cycloheximide, and puromycin inhibited control and picloram-induced growth and RNA and protein biosynthesis (^{14}C-labeled precursor incorporation). Since interpretation of the results of such precursor incorporation experiments assumes specificity of action of selected inhibitors of RNA and protein biosynthesis, a brief discussion of the action of three of the inhibitors is included.

Actinomycin D is well known as an inhibitor of RNA synthesis in higher plants as well as in animals and bacteria (26, 39, 65, 82, 84, 105, 116), but little is known about other possible effects of this antibiotic on plant processes. There are, however, good reasons for assuming its specificity of action, because a number of important processes are not affected by actinomycin D under conditions where RNA synthesis is inhibited. For example, actinomycin D inhibits normal RNA synthesis without affecting the multiplication and increase in infectivity of certain RNA viruses in higher plants (11, 135). At 7.0×10^{-5} M it did not significantly reduce the frequency of mitotic figures in onion (*Allium cepa* L.) roots for about 40 hr, though it caused a very sharp decrease thereafter (10). Respiration in potato discs which had reached a steady rate after excision, was not affected by 1.9×10^{-5} M actinomycin (39). So far all the data on higher plants suggest that actinomycin D acts by inhibiting DNA-dependent RNA synthesis, although in long treatment periods it might well show some secondary effects in some tissues.

Cycloheximide is generally known to inhibit protein synthesis, and recent work of Ellis and Macdonald (48) suggests that, in plants, it may indirectly produce such inhibition by interference with energy transfer reactions. In their studies cycloheximide at 1 µg/ml stimulated the respiration and inhibited the inorganic ion uptake by red beet (*Beta vulgaris* L.) discs. The uptake of organic compounds, on the other hand, was unaffected. Key (83), however, concluded that cycloheximide does not appear to be a respiratory poison, since the ATP/ADP ratio was maintained or increased in tissues treated for

several hours.

Puromycin is an effective inhibitor of protein synthesis though high concentrations are required to accomplish a reasonable inhibition of protein synthesis and growth of excised plant tissues (21, 82, 118, 119). Since it has no effect on the established respiration in aged potato tuber discs (39) its inhibition of protein synthesis and growth can hardly be due to interference with the energy-producing system.

Actinomycin D, cycloheximide, and puromycin all inhibited the control and picloram-induced growth and RNA and protein biosynthesis in excised soybean hypocotyl and barley coleoptile tissue (Figures 12, 13, and 14). Since protein synthesis is dependent on production of mRNA, actinomycin D can be expected to block such synthesis effectively and thus to interfere with any processes requiring synthesis of enzymes (unless their synthesis is controlled by a form of RNA synthesized via an actinomycin-resistant pathway). The inhibition of the incorporation of ^{14}C-leucine into protein in soybean hypocotyl and barley coleoptile sections by actinomycin D in my experiments is fully in line with the observations of other investigators on the inhibition of synthesis of new respiratory enzymes in potato discs (39) and of incorporation of amino acids into protein (82, 84, 111, 118, 158) by actinomycin D. The observed protein synthesis in the presence of actinomycin D must be due either to actinomycin-resistant RNA synthesis or to the activity of a relatively stable messenger or template RNA present in the tissue at the beginning of the experiment.

Cycloheximide, in addition to inhibiting protein synthesis, also inhibited RNA synthesis in soybean hypocotyl and barley coleoptile tissue. The possibility that inhibition of protein synthesis and thus of enzymes by cycloheximide may lead to inhibition of RNA synthesis should not be overlooked. The inhibition of ATP-incorporation into RNA in the present experiments was much less than the inhibition of leucine-incorporation into protein and probably is an indirect effect of cycloheximide. These results are fully in agreement with those of Key *et al.* (84) in similar experiments with cycloheximide on soybean hypocotyl.

Puromycin was not very effective in inhibiting protein synthesis and growth. Moreland *et al.* (111) found only 30 per cent inhibition of protein synthesis in soybean hypocotyl tissue by 50 µg/ml puromycin. A high concentration of puromycin, about ten times that used in the present studies, generally is required to obtain an inhibition of protein synthesis equivalent to that obtained here by actinomycin D and cycloheximide (Figure 14). If puromycin is slow in penetrating to the site of its action, this would, at least partially, explain its lower effectiveness in soybean hypocotyl and barley coleoptile sections. Since puromycin has a molecular weight of 472 and a low lipid solubility, it would not be expected to penetrate rapidly.

The data presented on actinomycin D inhibition of control and picloram-induced growth and RNA biosynthesis, and the work of Key and coworkers (84, 86), Masuda *et al.* (105, 106), and Nooden (116) support the view that the fraction of RNA synthesis which limits cell

enlargement may be relatively minor quantitatively. Quite likely, the synthesis or supply of rRNA or tRNA does not limit cell elongation and growth of excised plant tissues, whereas that of mRNA or some other type of RNA probably is limiting (84, 106, 116). It has been demonstrated (53, 105) that auxin favors the production of mRNA which becomes available for combination with free ribosomes to produce polyribosomes required for protein synthesis. My findings on picloram as an auxin are consistent with the idea that auxin acts by induction of some RNA synthesis, presumably mRNA (84, 86, 105, 106, 116), which results in the synthesis of new enzymes and ultimately modification of the cell wall, allowing cell expansion. There is now some definite evidence (112, 123) that auxins change the pattern of protein biosynthesis and that there are, in fact, new proteins synthesized in the presence of auxins. Norris (120) has presented evidence for such picloram-induced changes in cytoplasmic proteins of pea seedlings. It should be emphasized, however, that there are possible alternatives to the explanation presented above for auxin action. For instance, it has been proposed that auxin may act as an initiator of certain polypeptide chains, the hormone being bound to the N-terminal of some protein (5).

The inhibition of control and picloram-induced growth of excised soybean hypocotyl and barley coleoptile tissue by inhibitors of RNA and protein biosynthesis in these studies further supports the existing evidence that RNA and protein biosynthesis are essential for continued growth of excised plant tissues.

Contrary to the promotion observed at low concentration, picloram at a higher concentration drastically inhibited the growth and

RNA and protein biosynthesis of excised plant tissues. Might the inhibition effects be extensions of the same action of (auxin) picloram which invoked the promotion effects, or might they be related to separate actions? A mechanism similar to the two-point attachment theory of Foster *et al.* (55) for growth inhibition by auxin can be proposed for picloram action. These investigators based their assumption on the concept that auxin may act by attaching to some (enzymatic) entity in the cell. To stimulate growth, it must become attached at two positions. If two auxin molecules become attached at the same site, one molecule on each of the two positions, they would mutually inhibit by preventing the complete double attachment. Based on studies of the physical properties of active molecules, it has been suggested (170) that van der Waals and electrostatic forces are important in this auxin-receptor association. Picloram exhibits the particular structure i.e. a strong negative charge (arising from the dissociation of the carboxyl group) separated from a weaker positive charge on the ring (nitrogen of the amino group in the case of picloram) by a distance of approximately 5.5 Å, considered an essential requirement for auxin activity (170).

It is also possible that picloram determines the amount and types of protein synthesized; low picloram concentrations may induce the formation of those proteins (enzymes) required for active growth, but high concentrations may induce, in addition, formation of a protein which catalyses the synthesis of ethylene from its precursors (170) and thus inhibits growth of treated tissues. Further, the possibility of a direct effect of picloram at high concentrations on the collapse of cell protoplasm by plasmolytic water loss through the cell wall and/or

coagulation of cytoplasm (139) cannot be ignored.

Throughout my experiments I have noted that substances which promoted the incorporation of ^{14}C-ATP into RNA and of ^{14}C-leucine into protein commonly promoted uptake of the labeled precursors into the excised tissues, whereas those that inhibited incorporation also inhibited uptake, at the same time and under the same conditions. Essentially similar observations have been made in connection with other auxins and auxin-herbicides (81, 82, 89, 103) and antibiotics (81, 116, 119). The implication is that precursor uptake is in some way dependent upon continued synthesis of RNA and protein. An alternative explanation could be simply that uptake is a passive function of cell enlargement, and thus of promotion or inhibition of growth. Other data do not particularly favor the latter interpretation. For instance, when picloram approximately doubled the growth of barley coleoptiles it promoted the uptake of precursors only by 20 to 25 per cent. Since picloram influenced the uptake of ATP and leucine, it is possible that membrane permeability is altered. Such a suggestion has been made for picloram (90, 132) and other herbicides (103). Promotion or inhibition of uptake of precursors of RNA and protein synthesis by picloram may be physiologically significant, since cells of an intact plant must be capable of concentrating their required nutrients from rather dilute phloem and xylem contents.

Interpretation of the results obtained in excised tissue assays is complicated by factors such as the concentration of the herbicide at the site(s) of action as influenced by concentration of the herbicide

in the external medium and the length of incubation period; the possibility of involvement with indirect metabolic factors such as synthesis of precursors; interruption of the energy balance; species selectivity; possible effects on specific enzymes. To avoid some of these complications, future research should consider cell-free systems, postulated to be susceptible to this herbicide.

Excised tissue assays probably are not suitable for providing an indication of species selectivity based on biochemical mechanisms. Degradation or alteration of herbicide structure may not occur in the relatively short treatment intervals involved in excised tissue experiments. In addition, in RNA and protein biosynthesis studies only selected plant parts were involved. For example, shoot and root tissues were excluded, and none of the tissues was highly meristematic. Thus, many sites and tissues in which different responses could be obtained were eliminated in these experiments. Consequently, the status of RNA and protein constituents as measured following herbicide treatment of intact seedlings and plants with responses measured herein by excised tissue assays cannot be directly related. Even though nonmeristematic tissue was involved in these experiments, effects on DNA metabolism cannot be excluded from consideration (111). There is a possibility that the configuration of the DNA template is altered by picloram, which in turn would limit RNA and protein biosynthesis. From the results of this study on RNA and protein metabolism, and on the basis of proposals made by Hanson and Slife (66) for the action of auxin-herbicides, it is reasonable to assume that picloram-induced abnormalities in meristematic tissues (where most of the picloram is accumulated in

susceptible species following foliar or root application) lead to aberrant growth of susceptible plants. The immediate cause of death is physiological disfunction of leaves and roots brought about by abnormal growth in picloram-treated plants. The 'freezing' of the apical meristems results in cessation of new leaf and root production. Plants succumb because the abnormal growth reduces photosynthesis and normal phloem transport and the roots become unable to properly carry out their functions of salt and water absorption.

C. Uptake, Translocation, and Metabolism of Picloram

Excised soybean hypocotyls, barley coleptiles, barley leaf sections and Canada thistle leaf discs took up picloram from a liquid medium and, in the experimental period, only barley leaf sections accumulated it to a concentration higher than that of the external solution. A phase of net loss of radioactivity from excised segments immersed in a solution of ^{14}C-picloram is a distinctive feature of the 'Type I' (unstable accumulation) mechanism of uptake proposed by Venis and Blackman (165, 166) for auxinic-herbicides such as 2,3,6-TBA, 2,4-D, and 2,4,5-T. Since picloram has strong growth regulator properties, it is reasonable to suggest that the uptake and accumulation of picloram by excised plant tissues proceeds by a mechanism similar to that proposed by Venis and Blackman (165, 166). My results are in agreement with those of Baur *et al.* (16, 154) who suggested a similar mechanism for uptake of picloram by potato tuber discs.

Actinomycin D and cycloheximide inhibited the uptake of picloram by excised plant segments. To my knowledge there are no reports in the literature on the effects of these inhibitors on uptake of plant growth regulators, but they have been shown to inhibit the uptake of other organic compounds (81, 116, 119) and inorganic ions (48). The implication is that RNA and/or protein synthesis is in some way, directly or indirectly, related to the uptake of picloram by excised plant tissues. One such way could be simply through inhibiting the growth of these tissues and thus influencing the passive uptake of picloram.

High temperature coefficients (from 1.6 to 2.5 in the range of 5° to 35°C) and the marked response to ATP and DNP (Figure 16) are characteristics which specifically indicate an active or metabolic component in the entry of picloram into excised soybean hypocotyl and barley coleoptile sections. Evidence for initial entry of auxins such as IAA (37) and 2,4-D (75) by diffusion is suggestive of a passive uptake phase for picloram also. These lines of evidence strongly suggest that there are both passive and active components involved in the uptake of picloram, possibly an initial entry by passive diffusion and then immediate involvement of an active metabolic action. A similar suggestion has been made by Isensee *et al.* (73) for picloram uptake by oat and soybean seedlings from nutrient solution. Metabolic processes also could have an indirect effect on uptake by influencing cytoplasmic viscosity and accumulation and binding of picloram in the tissue. These processes will influence the concentration gradient across the surface layers.

Translocation of picloram in Canada thistle, soybean, and barley plants was very rapid. One day after foliar application a considerable amount of it was translocated out of the treated leaf and moved both upward and downward in the stem. The herbicide was accumulated in the rapidly developing tissues at the shoot apex of Canada thistle and soybean, whereas it was evenly distributed in barley plants. Initial acropetal movement in the treated leaf was in the apoplast, but the accumulation of picloram in young growing leaves in a short time (146) indicates a 'source-to-sink' pattern of symplastic translocation in the assimilate stream (41), which fits well with the mass-flow mechanism of phloem transport. The occurrence of a small amount of label in untreated mature leaves suggests that some picloram migrated from the phloem to the xylem and then was transported acropetally in the transpiration stream. Subsequent export of picloram from these mature leaves via the phloem appeared to be limited. The observation that picloram is exuded in Canada thistle (146) and other plant species (72, 133) in both soil and water cultures is in accord with the suggestion of Crafts and Yamaguchi (42) that the ability of a herbicide to leak from phloem to xylem relates to its ability to be exuded by roots. Thus, in cases where leakage into the transpiration stream occurs, leakage into the external medium from the roots also can take place.

My observations on translocation of picloram following foliar treatment do not suggest any direct inhibition of phloem transport in spite of an inhibition of assimilate transport by high concentrations of picloram in Canada thistle, soybean, and corn (Figures 6, 7, and 8, respectively) and in grape (97).

Theoretically, root-absorbed radioactivity should be evenly distributed in the plant, if it is freely mobile. However, the highest concentration of picloram was accumulated in the rapidly developing leaves at the shoot apex of Canada thistle and soybean after uptake by the roots. This type of accumulation following root uptake indicates a rapid redistribution of the herbicide with the assimilate flow in the plant. Most of the picloram that had entered the older leaves appeared to be retained by them. Reid and Hurtt (131) concluded that picloram taken up by bean roots bypassed mature leaves in its upward translocation. Their conclusion is open to question, however. A more probable explanation of their data is that picloram rapidly moved into mature leaves via the transpiration stream and then was immediately exported again via the phloem, with little retention in the leaves.

Since accumulation of ^{14}C-picloram after foliage or root application in Canada thistle and soybean is greatest in the parts farthest from the region of application, particularly in the young growing points which act as metabolic sinks, it seems that the susceptible regions of the plants are the metabolically active regions. The observed pattern of distribution of picloram corresponds well with the visible injury symptoms in plants after foliage or root application of picloram.

Differential absorption, translocation, and metabolism among species is a possible mode of herbicide selectivity. The extent of absorption and the pattern of subsequent distribution of picloram was different in Canada thistle, soybean, and barley plants. Absorption of picloram by Canada thistle and soybean was much faster and more complete

than that by barley plants following foliage application. Such differences in absorption and in the distribution patterns of picloram after shoot or root uptake undoubtedly contribute greatly to the expression of its selective action in grass and broad leaf species.

Evidence was obtained that picloram forms conjugates with plant constituents, in excised leaf tissues of barley and Canada thistle. Since there was no indication of such conjugation in similarly treated soybean hypocotyl and barley coleoptile sections the results cannot be attributed simply to microbial contamination. Because less than 1 per cent of the total activity was associated with macro-molecules such as proteins and nucleic acids (TCA-, phenol-, and ethanol-insoluble material) and the conjugates gave a positive reaction with benzidine for sugar, it is suggested that picloram in excised leaf tissues is conjugated mainly with sugar(s). The significance and implications of this observation, however, at present are not known.

Although differential rates of decarboxylation of picloram were observed in the different plant species tested, it would be misleading to attribute much importance to this because of the extremely low over-all rate of decarboxylation. The rate of $^{14}CO_2$ evolution from cotton plants 15 days after treatment with ^{14}C-picloram was negligible in the experiments of Meikle *et al.* (107). Sargent and Blackman (136), on the other hand, attributed picloram losses from bean leaf discs to decarboxylation, but they did not substantiate their explanation with $^{14}CO_2$-collection data.

The limited amount of information on the fate of picloram applied to plants is contradictory. Picloram does not seem to be rapidly immobilized or degraded by plants (72, 107). In the present studies, no indication of picloram metabolism in Canada thistle, soybean, and barley plants was found. It is possible, however, that degradation products of picloram were present in ethanol extracts and subsequently were lost in the purification procedures needed before the extracts of intact plants could be chromatographed. In excised tissue experiments no such purification steps were needed. Physiological changes such as an increase in certain enzyme(s) in excised leaf sections of barley and Canada thistle in the presence of light may be responsible for the conjugation of picloram with plant constituents observed in these tissues. The results of my experiments confirm the earlier reports (2, 3, 70, 107) of the persistent nature of picloram in plants, which makes this chemical a very potent herbicide.

The results reported in this thesis clearly indicate that differences in absorption and in distribution patterns of picloram after shoot or root application, together with differential effects, direct or indirect, on nucleic acid and protein metabolism, photosynthesis, and photosynthate translocation all play an important role in the susceptibility or resistance of the species studied to this herbicide. Further research on picloram aimed at pinpointing more exactly its site of action should include extensive investigations of enzyme systems concerned with intermediary metabolism, respiration, and photosynthesis. The subject of nucleic acid and protein metabolism merits detailed investigation, for the aberrant nucleic acid metabolism of sensitive

species is thought to underly death (30, 66, 87). Clearly, no full explanation of auxin-herbicidal action will be forthcoming until the biochemistry of nucleic acid metabolism in response to auxins can be adequately studied *in vitro*.

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