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Synthesis of Chymotrypsin Inhibitor I Protein in Potato Leaflets Induced by Detachment[1]

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Abstract. **Chymotrypsin inhibitor I is a protein that can be induced to accumulate in potato leaflets within hr in the light when leaflets are detached from intact plants and supplied with water. This increase in inhibitor I is not accompanied by an increase in all proteins in the detached leaflets. The accumulation is polar, proceeding from the base of the leaflets toward the tip. Low inhibitor I levels can be effectively maintained in detached leaflets by supplying them either water in the dark or solutions of indole acetic acid or 2,4-dichlorophenoxy acetic acid in the light. Inhibitor I from leaflets is not identical to inhibitor I isolated from potato tubers as determined by immunochemical analyses.**

In recent investigations (1, 2) of the occurrence of a proteinaceous inhibitor of chymotrypsin obtained from potato juice (3, 4) (called inhibitor I), it was observed that the protein could be induced to accumulate in leaflets of young potato plants by excising either the apex or newly formed stolens, or both. These studies also revealed that the inhibitor could be detected at various growth stages in nearly all of the tissues of the potato plant, and that its presence coincided with establishment and maintenance of meristematic tissue. In aging detached tubers, inhibitor I concentrations formed an increasing gradient from the stem end to the apex. In growing plants inhibitor I concentrations in aerial tissues increased preceding rhizome or tuber formation and subsequently decreased as it accumulated in the new tubers.

In the present communication it is reported that detached leaflets supplied with water commence a light-dependent accumulation of inhibitor I. This accumulation of protein is in contrast to the well-documented, extensive degradation of proteins that normally occurs in leaflets upon detachment. The evidence indicates that inhibitor I protein may be one of a few proteins accumulated specifically in response to leaflet detachment. It is apparently resistant to the degradation processes that are occurring within the detached leaflets. The evidence also shows that the protein from leaflets is not identical to the protein isolated from tubers.

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Materials and Methods

Five times crystallized chymotrypsin inhibitor I from potato juice was prepared as described by Balls and Ryan (4). Rabbit anti-inhibitor I serum was obtained from rabbits injected subcutaneously with 5 times crystallized inhibitor I emulsified in complete Freund's aduvant as previously described (2). Control serum was collected from the rabbits before injection. Using the method of Ouchterloney (6) anti-inhibitor I serum was reacted with either purified inhibitor I or crude potato tissue juice and gave in both cases a single precipitin line (2). Control serum did not react.

Inhibitor I concentrations in crude or centrifuged tissue extracts were determined by a rapid sensitive immunological method described previously (1, 8) based on the radial diffusion of antigens in agar gels containing antibodies.

Potato plants grown from true seed were used between 40 to 90 days after planting. Leaflet tissue was ground with a mortar and pestle and the juice collected by squeezing the macerate with a hand garlic press. The juice was centrifuged at 144,000*g* for 2 hr at 25° using a Spinco Model L centrifuge. The clear supernatant was drawn off using a Pasteur pipet. Inhibitor I concentration in the juice before and after centrifuging did not change.

In experiments using detached leaflets, only terminal leaflets from the middle and upper leaves of plants were used. The leaflets were placed with their petioles in small vials containing distilled water or 1 mM lysine. Incubation of the detached leaflets was carried out in a growth chamber under diurnal light (14 hr day, 1500 ft-c) with a temperature range of 18° in dark to 24° in the light, or in continuous light or dark at 24°.

Terminal leaflets low in inhibitor I were selected empirically. To this end, the juice from the first lateral twins was tested. If its concentration was less than 50 mg inhibitor I per ml the nearest terminal leaflet was considered suitable for use. This procedure was based on the following observations. The concentration of inhibitor I was determined in the juices of the terminal leaflets and in the nearest sets of lateral twin leaflets on each leaf, taken from a randomly selected sample of leaves from several plants. The results are shown in table I. While juice from different plants exhibited varying levels of inhibitor I, a definite pattern of concentration was seen in leaflets from individual leaves. The youngest leaflets, nearest the main stem, showed a higher concentration of inhibitor I than the first lateral twins or the terminal leaflet. Among the samples represented in table I, those underscored would have been selected.

Immunoelectrophoresis was carried out in 3 ml 1 % agar containing 0.1 M veronal buffer, pH 8.2, supported on 2.5 cm × 7.5 cm glass slides. Thymerosol at 1:10,000 dilution was used as a preservative in the gels. Electrophoresis was carried out for 3 hr at 12 volts per cm and 50 ma. After electrophoresis a lengthwise strip, 2 mm wide, was removed from the agar gel parallel to the direction of migration. Anti-inhibitor I serum, diluted 1:4 with the veronal buffer was added to the trough. After at least 24 hr incubation at room temperature, in a humid atmosphere, precipitin lines were photographed.

Protein was determined by the method of Lowry (7). Three times crystallized inhibitor I was used as a standard.

Radioactivity in total protein was determined by counting the precipitates formed in hot 5 % trichloroacetic acid. Precipitations were performed through the addition of 20 volumes of 5 % trichloroacetic acid at 80° to leaflet juice previously clarified by centrifugation at 144,000*g* for 30 min. The hot mixtures were centrifuged at 200*g* for 2 min using a clinical centrifuge. The precipitates were resuspended in hot 5 % trichloroacetic acid and centrifuged a total of 5 times. The precipitates were finally collected on 40 μ. Millipore filters, washed with 10 ml hot 5 % and 10 ml distilled water, and dried at 70°. Samples were dispersed in 4 ml of Bray's (9) counting solution. Counting was performed using a refrigerated Packard Tri-Carb Liquid Scintillation Spectrometer, Model 3314.

Inhibitor I was selectively precipitated from centrifuged leaflet juice using rabbit anti-inhibitor I serum. An equal mixture of juice and anti-inhibitor I serum (usually 0.1 ml each) was incubated in the refrigerator for 1 hr and centrifuged. The precipitates were suspended in 0.5 ml of 0.1 M veronal buffer, pH 8.2, containing 0.9 % NaCl, pelleted by centrifugation and resuspended and centrifuged 5 times in the buffer. After the final wash the precipitates were filtered on 40 μ Millipore filters, washed with 10 ml distilled water, dried at 70° and counted as described for the trichloroacetic acid precipitates. Serum, taken from the rabbit before injection of inhibitor I, was treated with the potato leaflet juice as a control. No precipitates formed upon treatment of leaflet juices with control serum.

Table I. *A Comparison of Inhibitor I Concentration in Terminal, First Lateral and Second Lateral Leaflets From Leaves of* Solanum tuberosum

Leaves were selected at random from several different plants.

Relative petiole position on plant	Second lateral	Leaflets first lateral[1]	Terminal
	μg inhibitor I/ml juice		
	82	50	*30*
Upper stem	78	44	*38*
near apex	120	80	61
	130	105	162
	120	44	*33*
	172	77	44
Middle stem	490	440	360
	...	0	*0*
	90	50	24
Lower stem	44	0	*25*
near soil level	...	253	140
	445	530	490

[1] Twin laterals nearest terminal leaflet.

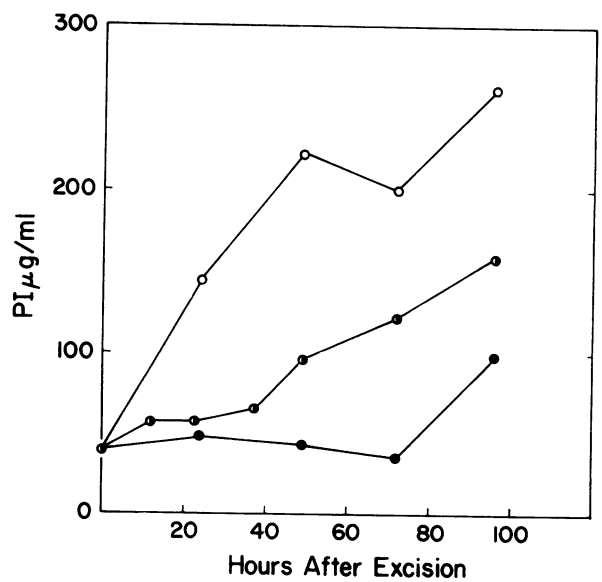


FIG. 1. Induced accumulation of inhibitor I (PI) in juice of excised potato leaflets. Matched young potato leaflets were excised at 0 hr and supplied with water in constant light (-○-○-); 12 hr day-night cycle (-◗-◗-); and constant darkness (●-●-). Juice from leaflets was tested for inhibitor I concentration at the times indicated by the radial diffusion method. Details are described in the Experimental section.

Results

As shown in figure 1, detachment of leaflets led to an increase of inhibitor I accumulation that was more rapid in continuous light than under diurnal conditions. Leaflets kept in continuous darkness did not exhibit any significant increase in the concentration of inhibitor I up to 72 hr.

In order to determine whether the leaflets would incorporate exogenously supplied lysine into inhibitor I protein, detached leaflets were fed 0.001 M uniformly labeled lysine ^{14}C (pH 7.0) containing a specific radioactivity of 1.49×10^5 dpm per μmole. Four matched leaflets were incubated at 24° in continuous light for 48 hr. As controls 1 set of 4 leaflets was incubated in the dark in 0.001 M lysine ^{14}C and 4 leaflets were incubated in the light without lysine ^{14}C. To the juice of the latter, after the induction period of 48 hr, was added lysine ^{14}C equal to twice the dpm taken up into the juice of the leaflets incubated with lysine ^{14}C in the light. These controls were run to determine (1) the extent of incorporation of lysine ^{14}C into leaflet juice in the dark when little or no inhibitor I was being synthesized, and (2) the extent of nonspecific adsorption of free lysine ^{14}C to trichloroacetic acid and antigen-antibody precipitates. Every 12 hr during the labeling time all leaflets, were transferred to fresh lysine ^{14}C solutions in order to minimize bacterial contamination. After incubation for 48 hr petioles were removed and discarded and the juice was recovered from leaflets by grinding them with mortar and pestle and expressing the macerate through a hand garlic press. The juice was clarified by centrifuging at 144,000g for 30 min and the precipitates were discarded. Labeled material present in the leaflets in both light and dark experiments was found to be completely soluble since centrifugation of the leaflet juice at 144,000g for 1 hr did not reduce the total dpm. In order to minimize affects of free lysine ^{14}C, small molecular weight material was removed in all 3 experiments by dialyzing 72 hr against several changes of 0.9 % NaCl containing 1:10,000 thymerosol. Dialysis removed approximately 85 % of label from the light and dark lysine ^{14}C supplied leaflets and 99.9 % of the lysine ^{14}C from the control experiment.

The dialyzed juices were tested for (a) the quantity of inhibitor I by the radial diffusion technique, (b) radioactive label in the total proteins insoluble in hot 5 %, and (c) label in inhibitor selectively precipitated with specific anti-inhibitor I serum. The data are summarized in table II.

The data suggest that if all of the potato leaflet proteins contain relatively the same percentage of lysine then 12.3 % of the newly synthesized protein in the light is inhibitor I protein, as compared with only 0.2 % in the dark. The radial diffusion assays show that 3.7 % of the total protein is inhibitor I. The dark treated leaflets contained only 1.0 % inhibitor I. These results indicate that detachment in light has specifically induced the accumulation of inhibitor I as newly synthesized protein. If some of the trichloroacetic acid precipitate material (table II) reflects an incorporation of lysine ^{14}C into the several hundred proteins already present in the leaflets, as well as inhibitor I, it is likely that only a few proteins are being selectively accumulated as a result of detachment in light. In 5 similar experiments incorporation of lysine ^{14}C into inhibitor I ranged from 9.7 % to 13.8 % of the total incorporation into trichloroacetic acid insoluble protein.

The Distribution of Inhibitor I in Detached Leaflets During the Accumulation Period. Large leaflets, approximately 10 cm from base to tip, containing low concentrations of inhibitor I, were detached from older tuberizing potato plants. The leaflets were supplied distilled water under continuous light of 1500 ft-c at 24°. At various times leaflets were dissected into several sections and their juice was expressed and tested for inhibitor I concentration. The results, shown in table III, clearly indicate that the accumulation of inhibitor I is polar, proceeding from the base of the leaflet toward the tip, but eventually including the entire leaflet.

Immunochemical Characterization of Leaflet and Tuber Inhibitor I. Earlier experiments using the Ouchterloney methods had shown that the inhibitor in aerial tissues of potato plants reacted with anti-inhibitor I serum to produce a single precipitin line.

Table II. *Summary of Incorporation of Lysine ^{14}C Into Protein and Inhibitor I in Detached Potato Leaflets*

A) Induction of inhibitor I	Total protein	Total inhibitor I[2]	% Inhibitor I
Treatment[1]	mg/ml	μg/ml	
Light	6.20	230	3.7
Dark	3.68	37	1.0

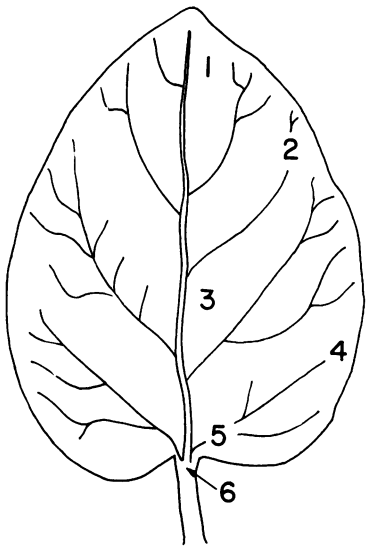
B) Incorporation of lysine ^{14}C into inhibitor I			
Treatment[1]	Trichloroacetic acid precipitate[3] cpm	Antibody-inhibitor I precipitate[3] cpm	% Incorporation into inhibitor I
Light	1.73×10^5	2.08×10^4	12.3
Dark	2.44×10^4	8.60×10^2	0.2

[1] Leaflets supplied with lysine ^{14}C for 48 hr in constant light or darkness. See Materials and Methods section for details.

[2] Radial diffusion method.

[3] Preparation of precipitates and counting procedures are described in the Materials and Methods section.

Table III. *The Polar Accumulation of Inhibitor I in Detached Potato Leaflets*



Leaf Position	Inhibitor I μg per ml Juice Days after Detachment			
	0	2	5	9
1	20	30	88	255
2	20	50	125	330
3	20	20	201	277
4	20	80	179	383
5	22	153	312	347
6	0	0	18	46

The line, however, was more diffuse than that of the tuber inhibitor I (1). A closer examination using more dilute serum has now revealed that the leaflet protein is definitely lacking antigenic determinants present in the tuber inhibitor I or purified crystalline inhibitor I. Figure 2 shows leaflet juice compared to tuber juice and to 3 times crystallized inhibitor I. The precipitin lines between tuber juice and purified inhibitor I are continuous, whereas both form definite spurs with the precipitin line from leaflet inhibitor I.

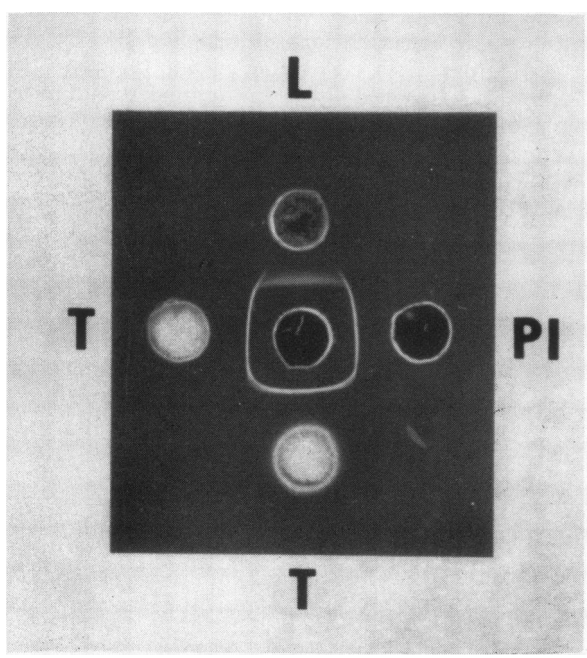


FIG. 2. Ouchterloney double diffusion analysis of the juice from various tissues of potato plants and purified inhibitor I. A 1:4 dilution of rabbit anti-inhibitor I serum was used in the center well. Wells T contained potato tuber juice; well L contained juice from an excised potato leaflet supplied with water in constant light for 48 hr; and well PI contained a solution of 125 μg/ml of thrice crystallized inhibitor I.

To examine this phenomenon further, leaflet juice, tuber juice, and 3 times crystallized inhibitor I were subjected to immunoelectrophoresis. Figure 3 shows juice from dark incubated and light incubated excised leaflets, and tuber juice, compared in each case to the crystalline inhibitor I. Juice from leaflets incubated in the dark did not react. Inhibitor I in juice from leaflets incubated in the light migrated toward the anode. In contrast, tuber juice inhibitor I and crystallized inhibitor I both migrated toward the cathode.

Effects of Indole Acetic Acid (IAA) and 2,3-Dichlorophenoxy Acetic Acid (2,4-D) and Kinetin on the Accumulation of Inhibitor I in Detached Leaflets. The preceding results indicated that detached leaflets can be used as a test system for studying factors that might be involved with the induced synthesis of inhibitor I. The effects of the plant hormone, IAA could thus be tested with this system. Key (10) had previously shown that IAA is a promoter of the synthesis of RNA and protein in soybean hypocotyl tissue, while Ockerse, *et al.*, (11) had demonstrated enzyme repression by IAA. It was therefore of interest to see if any effect of IAA on the accumulation of inhibitor I in detached leaflets could be observed. Experiments were performed using 4 potato plants in various stages of development and having differing amounts of inhibitor I naturally occurring in their leaflets. From these plants adjacent petioles, near the apex, were detached and numbered 1, 2, 3, and 4 counting the topmost petiole selected as No. 1. The terminal

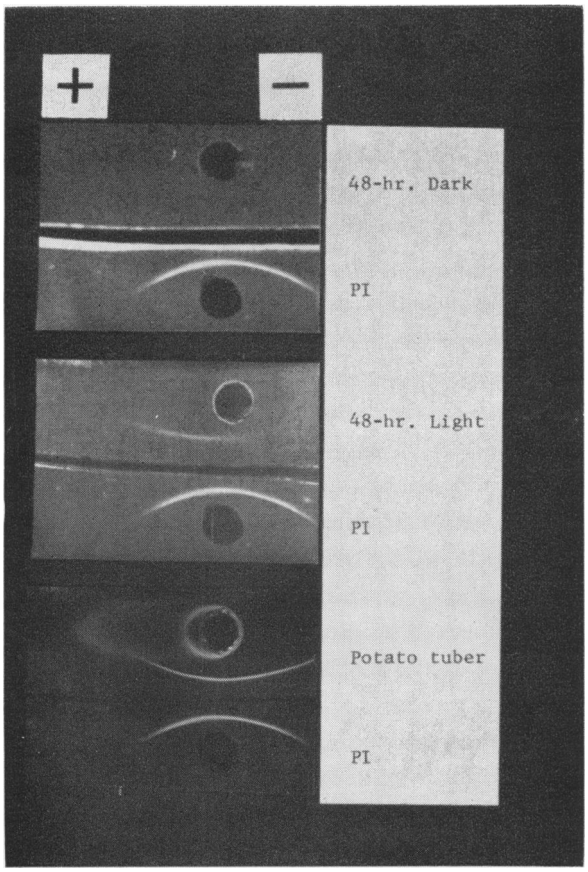


FIG. 3. Immunoelectrophoresis of juice obtained from dark and light treated excised potato leaflets and tuber juice, all compared to thrice crystallized inhibitor I. After electrophoresis for 3 hr the electrophoretograms were challenged with a 1:4 dilution of rabbit anti-inhibitor I serum in lengthwise slots cut in the agar gels. Details are described in the Experimental section.

leaflets of No. 2 and No. 3 petioles were fed either water or 1×10^{-4} M IAA. Terminal leaflets from No. 1 and No. 4 were tested as controls to determine initial levels of inhibitor I in the plants. Table IV summarizes the data. It can be seen from leaflets on petioles No. 1 and No. 4 that the different plants tested contained differing amounts of inhibitor I. A pattern of similarity in magnitude, but decreasing from the apex down is easily seen in each plant. It is interesting to note that an increase in inhibitor I concentration could be induced by leaflet detachment, but that IAA could prevent synthesis completely or nearly so only in plants initially containing relatively low concentrations of inhibitor I, *i.e.*, plants No. 1 and No. 2. In plants initially containing appreciable inhibitor I in leaflets IAA did not exhibit this pronounced effect, but only partially retarded the induction. These experiments suggest that if IAA is acting to repress the synthesis of inhibitor I, it is at a step preceding the actual translation of messenger RNA. These experiments (plants No. 3 and No. 4) also suggest that IAA is not activating a system capable of destroying inhibitor I.

The concentrations at which IAA was an effective inhibitor of the accumulation in detached potato leaflets was determined. The synthetic herbicide 2,4-dichlorophenoxy-acetic acid (2,4-D) was similarly studied. Table V shows the cumulative results of 2 experiments performed with matched leaflets of young plants. In each experiment 2 leaflets were used at each concentration. The results show considerable inhibition by IAA at 10^{-4} to 10^{-8} M and for 2,4-D at 10^{-4} and 10^{-6} M. Inhibition of accumulation of inhibitor I by these compounds is variable in degree depending upon the leaflets tested. It was shown previously (table IV) that the effectiveness of IAA is considerably influenced by the physio-

Table IV. *Effect of Indolecactic Acid on the Synthesis of Inhibitor I Protein in Detached Leaflets of Potato Plants*

Plant	Terminal leaflet from petiole	Treatment	Inhibitor I in leaflet juice
No.	*No.*		*μg/ml*
1	1	None	72
	2	H_2O, 48 hr	330
	3	10^{-4} M IAA, 48 hr	98
	4	None	61
2	1	None	20
	2	H_2O, 48 hr	375
	3	10^{-4} M IAA, 48 hr	0
	4	None	0
3	1	None	112
	2	10^{-4} M IAA, 48 hr	225
	3	H_2O, 48 hr	245
	4	None	92
4	1	None	220
	2	10^{-4} M IAA, 48 hr	330
	3	H_2O, 48 hr	400
	4	None	138

[1] Adjacent petioles near apex of plant. Numbered from uppermost down.

Table V. *Inhibition of Accumulation of Inhibitor I in Detached Potato Leaflets by IAA and 2,4-D in Constant Light for 48 Hr*

Two matched leaflets were used at each level of IAA or 2,4-D in each experiment. The leaflets were supplied with solutions from 16 ml plastic capped vials, painted black, through the petioles which reached the solutions by way of small holes in the caps. At 24 hr, all vials were emptied and refilled with fresh solutions.

Conc	Inhibitor I Accumulated in 48 hr	
IAA	Expt No. 1	Expt No. 2
M	*μg/ml juice*	
0	165	280
10^{-4}	71	103
10^{-6}	52	134
10^{-8}	74	88
10^{-10}	165	167
2,4-D		
M		
0	222	168
10^{-4}	87	53
10^{-6}	93	43
10^{-8}	91	181
10^{-10}	166	180

logical state of the leaflet. The results of table V do however reflect the concentrations at which these compounds are most effective as inhibitors of the accumulation.

Experiments were also conducted to find if kinetin would affect the accumulation of inhibitor I in detached leaflets in the presence or absence of IAA. Table VI indicates that kinetin had little or no effect.

Discussion

The observation that inhibitor I can be induced to accumulate in leaflets within hr in the light by excising them from the potato plant was a logical consequence of our earlier studies (1, 2). We had observed that inhibitor I protein concentrations increased rapidly in leaflets or stems when meristematic tissues of the plants, such as apices or rhizomes, or both, were removed. The meristematic tissue was apparently influencing the levels of inhibitor I in the leaflets. By removing leaflets from this influence through detachment, in contrast to removing the meristems themselves, a rapid accumulation in the leaflets was observed. It can be seen from figure 1 that the accumulation was more effective in constant light than in diurnal light, or in total darkness where accumulation was not significant. It is not yet known how the light affects accumulation, or what intensities are required for the maximum effect. Studies concerning the optimal wavelengths and intensities are now in progress.

Table VI. *Effects of IAA and Kinetin on Accumulation of Inhibitor I in Detached Potato Leaflets*

Treatment of detached leaflet	Inhibitor I in leaflet juice (48 hr induction period)
	μg/ml
Total darkness	0
Continuous light	307
Continuous light + 10^{-4} M IAA	53
Continuous light + 10^{-4} M Kinetin	260
Continuous light + 10^{-4} M IAA + 10^{-4} M Kinetin	53
Intact leaflet - no treatment	0

The maximum accumulation of inhibitor I under conditions shown in figure 1 is about 1 % of the total protein present. The amount of accumulation between matched leaflets was usually quite similar but the percent total protein varied among leaflets due to varying endogenous protein levels. This percentage has been found to vary from less than 1 % to over 3 % depending upon the particular leaflets tested. In general, older detached leaflets accumulated inhibitor I more slowly, but ultimately just as effectively, as younger leaflets. This can be seen by comparing experimental data from figure 1 and table III. Data in figure 1 was obtained using small young growing leaflets whereas data in table III was obtained using large older mature leaflets.

The pattern of polarity of accumulation in the leaflets, increasing at the base and proceeding toward the tip indicated that all cells of the leaflets are not equivalent in their capacities to accumulate inhibitor I within a given time of induction. This polarity is not yet understood, but may be a result of the same factors that influence the differences in rates of accumulation of inhibitor I between young and old detached leaflets.

In experiments in which lysine ^{14}C was fed to the detached leaflets in the light it was demonstrated that a significant part of the accumulated protein incorporated exogenously supplied lysine ^{14}C. The supplied label apparently had easy access to the pool of amino acids from which the new inhibitor I was synthesized. The high percentage of label in inhibitor I (12.3 %) with respect to all labeled proteins suggests that inhibitor I is being synthesized in detached leaflets in light preferentially with respect to other proteins in the leaflets. The data from table II A and figure 1 support this hypothesis.

Data presented here and in previous reports (1, 2) indicate a relationship between meristematic tissue and leaflet processes concerned with specific protein synthesis. The relationship could be one involving small molecules (hormones) and the control of specific genes. Another explanation of the induced accumulation is that detachment inhibits a light mediated transport of metabolites or of the inhibitor I itself away from the leaflets. The ob-

servation that IAA can inhibit the accumulation would support the hypothesis that hormones may be involved and would implicate IAA as a possible control factor. However, IAA could be acting indirectly by influencing a system within the leaflets that produces yet another small molecule that is normally exported to meristems, but that is an active inducer of inhibitor I when leaflets are detached or when meristems are removed. It was of interest that IAA did not significantly inhibit accumulation of inhibitor I in leaflets already making the protein. Table IV data imply that IAA was not simply inducing or activating a system capable of destroying inhibitor I, but was probably affecting protein synthesis at some step before messenger RNA for inhibitor I was used. When inhibitor I was being actively synthesized IAA could not shut it off. Unless messenger RNA for inhibitor I was very long lived, or unless a stable inducer was present acting independently of IAA, a more pronounced effect would have been expected if IAA was acting directly at the gene level.

The immunological and immunoelectrophoretic analyses have shown that the inhibitor I protein synthesized in the leaflet is not identical to that made in the tuber. The differences were seen in both antigenic determinants and electrophoretic mobility. The differences might be explained if some basic functional groups of inhibitor I were substituted with a neutral or anionic group or modified by complexing to an endogenous leaflet substance. Alternately the leaflet inhibitor might be modified through proteolysis by a leaflet proteinase. However, incubating tuber inhibitor I with various leaflet extracts did not result in formation of a modified leaflet inhibitor I protein, determined immunochemically. To date no evidence has been found for the existence of tuber inhibitor I in leaflets, or for leaflet inhibitor I in tubers.

If the differences in the tuber and leaflet proteins are not due to cellular modification after synthesis then the possibility of the existence of duplicate genes for inhibitor I in the potato plant will have to be considered. Since 1 form of the protein is synthesized in green leaves and only in the light while the other form of the protein is found in the tuber, underground in the dark, differences in the metabolism of the 2 tissues may require the presence of 2 genes, perhaps under different controls within the plant. Experiments to further understand the relationship between inhibitor from leaflets and tubers are in progress.

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Literature Cited

1. Ryan, C. A., O. C. Huisman, and R. W. Van Denburg. 1968. Transitory aspects of a single protein in tissues of *Solanum tuberosum* and its coincidence with the establishment of new growth. Plant Physiol. 43: 589–96.
2. Ryan, C. A. and O. C. Huisman. 1967. Chymotrypsin inhibitor I from potatoes: a transient protein component in leaves of young potato plants. Nature 214: 1047–49.
3. Ryan, C. A. and A. K. Balls. 1962. An inhibitor of chymotrypsin from *Solanum tuberosum* and its behavior toward trypsin. Proc. Natl. Acad. Sci. U.S. 48: 1839–44.
4. Ryan, C. A. 1966. Chymotrypsin inhibitor I from potatoes: Reactivity with mammalian, plant, bacterial, and fungal proteinases. Biochemistry 5: 1592–96.
5. Balls, A. K. and C. A. Ryan. 1963. Concerning a chymotryptic inhibitor from potatoes and its binding capacity for the enzyme. J. Biol. Chem. 238: 2975–82.
6. Ouchterloney, O. 1949. Antigen-antibody reactions in gels. Acta Pathol. Microbiol. Scand. 26: 507.
7. Lowry, O. H., N. J. Rosbrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265–90.
8. Ryan, C. A. 1967. Quantitative determination of soluble cellular proteins by radial diffusion in agar gels containing antibodies. Anal. Biochem. 19: 434–40.
9. Bray, G. A. 1960. A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. Anal. Biochem. 1: 279–85.
10. Key, J. L. 1964. RNA and protein synthesis as essential processes for cell elongation. Plant Physiol. 39: 365–70.
11. Ockerse, R., B. I. Siegel, and A. W. Galston. 1966. Hormone-induced repression of a peroxidase isozyme in plant tissue. Science. 151: 452–53.