INCREASED TEMPLATE ACTIVITY IN CHROMATIN FROM CADMIUM CHLORIDE TREATED PEA TISSUES

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SUMMARY

Increases in the synthesis of the isoflavonoid, pisatin, and the activity of phenylalanine ammonia lyase (PAL) are induced in excised pea pods by low concentrations ($5X10^{-4}M$) of $CdCl_2$. The induction of pisatin synthesis and PAL are suppressed if RNA-synthesis-inhibiting concentrations of 6-methyl purine, actinomycin D or ∝-amanitin are applied within 1 h of inducer application. Cycloheximide (0.1 mg/ml) blocks the induction of these responses if applied to tissues within 6 h after inducer application. Within 1 h after $CdCl_2$ ($5X10^{-4}M$) is applied to pods there is an increase in the rate of synthesis of all sizes of RNA as well as an increase in the template activity and dye binding capacity of pea chromatin. The results support the hypothesis that conformational changes in DNA are associated with the induction process.

Precise biological effects of cadmium are difficult to decipher because of the multiplicity of sites (1) within the cell which react with heavy metals. Low concentrations of heavy metal salts (2-4) such as $CdCl_2$, $CuCl_2$, $HgCl_2$, and $CrCl_3$ can mimic plant pathogenic organisms in inducing the production of high concentrations of the "phytoalexin", pisatin, in peas as well as the production of other isoflavonoid phytoalexins in various plants (5,6). The antifungal properties of phytoalexins are believed to be essential in protecting legumes from certain plant pathogens (7). In general, the induction of phytoalexin production in peas (7) and other plants (8) has been a paradox in that certain compounds, regarded as inhibitors of RNA synthesis, actually stimulate RNA synthesis as well as massive increases in both pisatin and the activity of phenylalanine ammonia lyase (PAL), an enzyme in the biosynthetic pathway of pisatin (9). These gene-controlled responses in peas can also be induced by certain DNA-alkylating agents (7), base analogs (7), DNA-intercalating compounds (10), UV light (260 nm) (11), UV light (366 nm) in combination with plant psoralen compounds (12), fungal

mycelial extracts (2,4) and DNA-complexing basic molecules such as poly-L-lysine (13), spermine (10) and RNase (13). (Plant hormones, steriod hormones, numerous cytotoxic compounds and simple stimuli such as wounding are not effectors of these responses). Pisatin production is induced almost exclusively by compounds known (14-16) to change, in various ways, the conformation of DNA.

We have employed this well-defined induction system to test the effects of heavy metals, whose DNA conformation-changing properties are well documented (16-18). This report provides evidence that in pea tissues low concentrations of heavy metals increase pisatin synthesis, PAL activity, RNA synthesis and the template activity and actinomycin D binding sites of chromatin.

MATERIALS AND METHODS

Materials.--α amanitin was donated by TH. Wieland, Max-Plank-Institute. Actinomycin D-^{3}H was obtained from Schwarz-Mann, Orangeburg, N.Y.

Induction treatments.--Immature Alaska pea (Pisum sativum L.) pods (less than 2 cm long) were harvested while still enclosed in the blossom to minimize contamination. The pods (1 g/treatment) were immediately split and placed in covered sterile petri dishes.

Treatments (1.0 ml of inducer solution) were applied to the exposed endocarps of 1 g pod. Pods were then placed in the dark at 22°C. After 18 and 24 hour incubation periods PAL was assayed (13) and pisatin was quantitated (7) respectively, as described previously. Heavy metal solutions were sprayed on aseptically grown pea seedlings to induce pisatin production.

RNA synthesis.--The relative rates of RNA synthesis in induced and non-induced tissue were determined by pulse-labeling the pea tissue with orotic acid -6-^{14}C and orotic acid -5-^{3}H respectively. Orotic acid 6-^{14}C (3.0 µc) was applied to the endocarp surface of 3 g of split, immature pea pods 15 m prior to treatment with $CdCl_2$ ($6X10^{-4}$M). Another 3 g sample was labelled with orotic acid -5-^{3}H (30 µc) and subsequently treated (15 m later) with water. After 30 m the two samples were combined and the RNA was extracted at 60°C by the phenol method (19) and fractionated by linear (5-40% sucrose) density gradient

centrifugation. Centrifugation was performed in a SW-50-L rotor for 18 h at 32,000 rpm at 0 to 4 C. The ratios were corrected for counting efficiencies and relative counts administered as described previously (7). Control ratios were obtained by dual labeling two sets of water-treated pods.

RNA Polymerase Assay: Pea chromatin was extracted according to Huang & Bonner (20) except that $MgCl_2$ was deleted from the "grinding medium". The H_2O and $CdCl_2$-treated tissues were thoroughly washed 7 times in 10X volumes of sterile water to remove external $CdCl_2$ prior to chromatin extraction. The extracted chromatin was run through two density gradient purifications and was quantitated (21) on the basis of actual µg DNA. The E. coli polymerase used in these assays was the "pooled phosphocellulose peak" described by Burgess (22) which does not contain sigma factor.

The rate of RNA synthesis was measured by determining the incorporation of UTP-^3H into a trichloroacetic acid-insoluble fraction. Incubation was carried out for 10 m at 37°C in a final volume of 0.2 ml containing 10 µmoles Tris-HCl (pH 7.5), 1.0 µmole $MgCl_2$, 0.25 µmole $MnCl_2$, 3 µmoles β-mercaptoethanol, 0.04 µmoles of ATP, GTP and CTP, and 0.04 µmoles UTP-^3H (5µc), 1.0 µg DNA or 1.0 µg of chromatin DNA and 0.1 unit of E. coli RNA polymerase. Incubation was stopped by the addition of 5% trichloroacetic acid (with 0.1 mg/ml pyrophosphate) at 0 C. The mixture was filtered through a milipore filter (PHWP 02500) and the filter was washed 3 times with 15 ml of 5% TCA and twice with 15 ml chloroform. The filter was dried and the radioactivity measured in 10 ml of Triton-X based scintillation fluid in a Nuclear Chicago liquid scintillation counter.

Dye-binding of pea chromatin.--The actinomycin D binding capacity of pea chromatin isolated as above was determined according to the methods of Beato et. al. (23). Actinomycin D-^3H (1 µc/0.63 mg) was mixed with chromatin (the equivalent of 1 mg DNA in 2 ml Tris, pH 7.5) preparations from $CdCl_2$ ($5X10^{-4}M$) -treated and water-treated tissues. Each combination was stirred 15 m at 4°C and after an additional 15 m was layered on a 5-20% sucrose gradient and centrifuged for 15 h at 39,000 rpm in a SW 40 Spinco rotor. The respective

Table 1. Effect of metals on the levels of phenylalanine ammonia lyase (PAL) and pisatin in pea pod cells.

Chemical Applied	Conc.	PAL-nmoles Cinnamic Acid	Pisatin µg/g
$CdCl_2$	$1X10^{-3}M$	1,157 ± 46	107
$CdCl_2$	$5X10^{-4}M$	1,242 ± 28	156
$HgCl_2$	$1X10^{-3}M$	1,036 ± 101	123
$ZnCl_2$	$6X10^{-3}M$	345 ± 50	155
$NaCl_2$	$3X10^{-3}M$	102 ± 2	0
$MnCl_2$	$3X10^{-3}M$	89 ± 4	0
$MgCl_2$	$3X10^{-3}M$	91 ± 15	0
$CoCl_2$	$3X10^{-3}M$	146 ± 14	11
H_2O		90 ± 6	0
Actinomycin D	10 µg/ml	972 ± 114	365

One ml aqueous solution of each chemical was applied to the endocarp of the pods prior to being incubated in the dark at 22°C for 18 h (PAL assay) or 24 h (pisatin assay). PAL activity is expressed as nmoles cinnamic acid/g pod/h at 37°C. PAL and pisatin were assayed as described previously (7,13). Concentrations of metal salts tested ranged from $1X10^{-2}$ to $1X10^{-5}M$, only the results from optimal concentrations are included in this table.

pellets were employed for the determination of radioactivity bound to the template and for UV measurements. Also, aliquots of the resuspended pellets were layered on 6M $CsCl_2$ and centrifuged 38,000 rpm for 72 h in a Spinco Ti 50 rotor to establish that most of the bound Actinomycin $D^{-3}H$ was associated with fractions of the pure DNA and only a minimal portion of radioactivity occurs with the protein fractions in the upper part of the gradient.

RESULTS AND DISCUSSION

Increased amounts of pisatin can be induced when low molar concentrations

of the heavy metal salt are either applied to the endocarp surface of excised, immature tissue of pea pod halves (Table 1) or sprayed on intact pea seedlings. Cadmium chloride ($5X10^{-4}M$), $HgCl_2$ ($1X10^{-3}M$) or $ZnCl_2$ ($6X10^{-3}M$) increase the pisatin level of pea tissue from non-detectable (<3 µg) to more than 100 µg per g tissue in 24 h. Under the same conditions the metal salts, NaCl, $MnCl_2$ and $CoCl_2$ ($1X10^{-1}$ to $1X10^{-4}M$) were ineffective. Greater than 10 fold increases in PAL activity were obtained within 18 h after the application of $6X10^{-4}$ to $1X10^{-3}M$ solutions of $CdCl_2$ or $HgCl_2$. The activity of PAL extracted from non-induced tissues could not be increased by comparable concentrations of these metal salts in the reaction mixture. The greater than 10 fold increases in phenylalanine ammonia lyase were obtained only in the pod tissue of peas since apparently the inherent level of this enzyme is already maximal in seedling tissues. In general, increases in PAL and pisatin are detectable at 3 and 8 h, respectively, and peak levels are approached 18 and 30 h, respectively, after inducer application. These induction responses were suppressed by high concentrations of RNA-synthesis inhibitors such as 6 methylpurine (1 mg/ml), actinomycin D (0.6 mg/ml) or α-amanitin (1.0 mg/ml) if applied within 1 h of the application of the heavy metal. The protein synthesis inhibitor cycloheximide (0.1 mg/ml) inhibits these responses if applied within 6 h after application of the inducer.

Cadmium chloride ($6X10^{-4}M$) increases the rate of RNA synthesis in pea tissue as indicated by the experiments shown in Fig. 1. The stimulation appears to result from a differential rate of synthesis throughout the various sized species of RNA.

The cadmium chloride treatment was found to affect significantly the accessibility of the chromatin DNA to both RNA polymerase and actinomycin D. We compared the template activity of purified chromatin from $CdCl_2$ ($6X10^{-4}M$)-induced and non-induced pea tissue to evaluate the relative RNA synthesis potential *in vitro*. The data in Table 2 indicate that the template activity of chromatin extracted from $CdCl_2$ ($6X10^{-4}M$)-treated (1 h) tissue is consistently higher than that of chromatin extracted from H_2O-treated tissues. When the

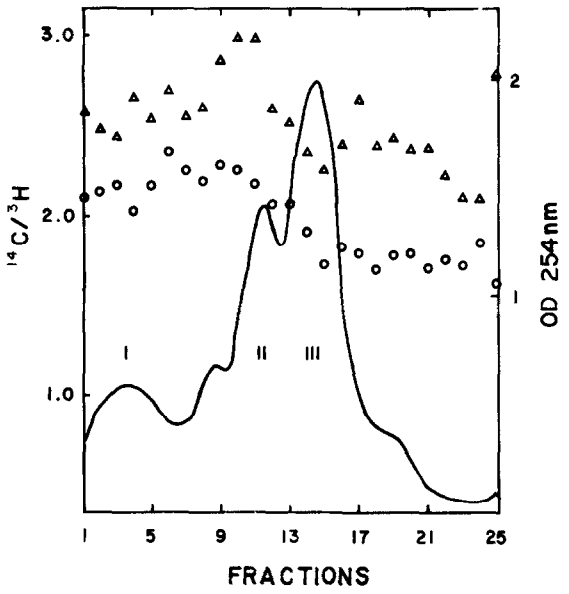

Fig. 1. Relative incorporation of radioactively labeled orotic acid into various fractions of RNA extracted from $CdCl_2$-treated versus water-treated pea pod tissue. Orotic acid 6-^{14}C (3 μc) was applied to the endocarp surface of 3 g of split, immature pea pods 15 m prior to treatment with $CdCl_2$ ($6X10^{-4}M$). Another 3 g sample was labeled with orotic acid -5-^{3}H (30 μc) and subsequently treated (15 m later) with water. After 30 m the two samples were combined and the RNA was extracted and fractionated by density gradient centrifugation (see Methods). The ratios (corrected for counting efficiencies and relative counts administered as described previously (7) dpm ^{14}C to dpm ^{3}H ($^{14}C/^{3}H$) are indicated by the triangles. The open circles indicate ratios obtained by dual labeling two sets of water-treated controls. The solid line indicates the optical density at 254 nm. I = 4S and/or 5S RNA; II = light ribosomal RNA; III = heavy ribosomal RNA.

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reaction mixture containing chromatin from H_2O-treated tissue was supplemented with $CdCl_2$ ($6X10^{-4}$ to $6X10^{-5}M$), polymerase activity decreased. Chromatin extracted from $CdCl_2$ and H_2O-treated tissues was also shown to differ in actinomycin D binding capacity. The chromatin containing pellet from the $CdCl_2$-treated

Table 2. Template activity of freshly prepared chromatin from H_2O or $CdCl_2$ induced pea pod tissue assayed with E. coli RNA polymerase.

Template	Deletions from Reaction Mixture	Labelled UTP-^{3}H incorporated (μμmol/10 min/mg DNA) Prep I	Prep II
H_2O-chromatin	-Enz	1,059	1,052
H_2O-chromatin		3,866	2,505
$CdCl_2$-chromatin		6,255	5,162
DNA		111,975	27,045
H_2O-chromatin	-Mg-Mn	451	--
$CdCl_2$-chromatin	-Mg-Mn	393	--
DNA	-Mg-Mn	364	0

Split immature pea pods (60g) were treated with H_2O or $CdCl_2$ ($6X10^{-4}M$) for 1 h and the chromatin was extracted according to Huang & Bonner (20) except that $MgCl_2$ was deleted from the grinding medium. Template activity of chromatin and DNA were determined as described in Methods. The chromatin preparations designated "Prep I" and "Prep II" were from different harvests of pea pods. The extraction and assay procedures were similar except that the Prep II extraction procedure was not interrupted before assaying. Also Prep I was assayed with enzyme in excess of the saturation level whereas Prep II was assayed with a 50% saturation level of the enzyme.

tissue complexed with 22% more actinomycin D-^{3}H than did the chromatin from H_2O-treated tissue.

The remarkable effects of cadmium in peas in increasing PAL activity, the rate of RNA synthesis, and the template activity and actinomycin D binding sites of pea chromatin suggest that this heavy metal is directly involved in the activation of gene-controlled responses. Since increases in RNA synthesis occur follow-

ing the application of low concentrations of either heavy metals or RNA synthesis "inhibitors" such as actinomycin D (7), we propose that no "paradox" (24, 25) exists in this induction system of pea tissue. These results suggest that the heavy metals have a specific stimulatory function in the cell at low concentrations at which other toxic effects are minimal. Similarly, the cellular action of low concentrations of DNA-specific compounds such as actinomycin D should be viewed both in terms of their physical effects (15) on the DNA of chromatin as well as their blocking effects on the transcription of DNA.

It is tempting to relate the increase in actinomycin D binding sites and the polymerase availability of chromatin from $CdCl_2$-treated tissue to the previously reported (1,16,17,18) effects of heavy metals on DNA *in vitro*. Cadmium^{2+} and Hg^{2+} ions which induce phytoalexin production are known to bind preferentially to the heterocyclic bases of DNA, disrupting the hydrogen bonding, changing DNA conformation and lowering Tm, the mean temperature at which thermal denaturation of DNA occurs. In contrast, other ions, including Na^{+}, Mn^{2+} and Co^{2+} which do not induce, appear to bind to the sugar-phosphate backbond, stabilizing the double-helical conformation and raising the Tm. Thus, one explanation for the action of cadmium in this report is that it could assist in the "melting-in process" (14) of polymerase initiation by lowering the Tm within certain regions of the DNA.

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REFERENCES

1. Vallee, B. L. and Ulmer, D. D., Ann. Rev. Biochem. 41, 91(1972).
2. Cruickshank, I. A. M. and Perrin, Dawn R., Aust. J. Biol. Sci. 16, 111(1963)
3. Perrin, Dawn R. and Cruickshank, I. A. M., Aust. J. Biol. Sci. 18, 803(1965).
4. Schwochau, M. E. and Hadwiger, L. A., Arch. Biochem. Biophys. 126, 731(1968).
5. Cruickshank, I. A. M. and Perrin, Dawn R., Phytopath. 2, 60, 335(1967)
6. Klarman, W. L. and Sanford, J. B., Life Sci. 7, 1095(1968).
7. Schwochau, M. E. and Hadwiger, L. A., Arch. Biochem. Biophys. 134, 34(1969).
8. Hess, S. L. and Hadwiger, L. A., Plant Physiol. 48, 197(1971).
9. Hadwiger, L. A., Phytochem. 5, 523(1966).
10. Hadwiger, L. A. and Schwochau, M. E., Plant Physiol. 47, 346(1971).

11. Hadwiger, L. A. and Schwochau, M. E., Plant Physiol. 47, 588(1971).
12. Hadwiger, L. A., Plant Physiol. 49, 779(1972).
13. Hadwiger, L. A. and Schwochau, M. E., Biochem. Biophys. Rs. Comm. 38, 683(1970).
14. Von Hippel, P. H. and McGhee, J. D., Ann, Rev. Biochem. 41, 231(1972).
15. Waring, M., J. Mol. Biol. 54, 247(1970).
16. Lazurkin, Yu. S., Frank-Kamenetskii, M. D. and Trifonov, E. N., Biopolymers 9, 1253(1970).
17. Sundaralingam, M. and Carrabine, J. A., J. Mol. Biol. 61, 287(1971).
18. Eichhorn, G. L. and Shin Y. A., J. Amer. Chem. Soc. 90, 7323(1968).
19 Ingle, W., Key, J. L. and Holm, R. E. J. Mol. Biol. 11, 730(1965).
20. Huang, R. C. and Bonner, J., Proc. U. S. Nat. Acad. Sci. 48, 1216(1962).
21. Burton, K., Methods of Enzymology XIIB, 163(1968).
22. Burgess, R. R., J. Biol. Chem. 244, 6160(1969).
23. Beato, M., Seifart, K. H. and Sekeris, C. E., Arch. Biochem. Biophys. 138, 272(1970).
24. Garren, L. D., Howell, R. R., Thomkins, G. M. and Crocco, R. M., Proc. U. S. Nat. Acad. Sci. 52, 1121(1964).
25. Thompson, E. B., Granner, D. K., and Tomkins, G. M., J. Mol. Biol. 54, 159(1970).