Biochemical Pharmacology, Vol. 20, pp. 3255–3261. Pergamon Press, 1971. Printed in Great Britain

INDUCED FORMATION OF PHENYLALANINE AMMONIA LYASE AND PISATIN BY CHLORPROMAZINE AND OTHER PHENOTHIAZINE DERIVATIVES*

LEE A. HADWIGER and ARNOLD R. MARTIN

Department of Plant Pathology and College of Pharmacy, Washington State University, Pullman, Wash. 99163, U.S.A.

(*Received* 11 *January* 1971; *accepted* 14 *April* 1971)

Abstract—Chlorpromazine and 16 other phenothiazine derivatives induced up to 11-fold increases in phenylalanine ammonia lyase activity and the *de novo* synthesis of up to 200 μg pisatin per g of pea pod tissue. The induction of phenylalanine ammonia lyase was dependent on new RNA and protein synthesis and was accompanied by increased synthesis of an array of cellular proteins.

MANY of the phenothiazine derivatives utilized as tranquilizers and sedatives have undesirable side effects (some of which may be caused by metabolites) such as skin rash, dizziness and jaundice.[1] The physiological occurrences which account for these conditions are not yet understood. These responses in animal systems may stem from increased synthesis of enzymes.

Recently, a series of drugs which have planar three-ring structures have been shown[2–4] to induce the enzyme, phenylalanine ammonia lyase (PAL; EC 4.3.1.5), and pisatin production in pea pod tissue.[5] Of this series, the antimalarial compound, quinacrine, excelled as an inducer of both responses. The phenothiazine derivative, chlorpromazine and its sulfoxide (a metabolic product of chlorpromazine in humans) utilized in the present study are chemically related to the acridine derivative, quinacrine. Both compounds have chlorinated three-ring structures and similar aliphatic side chains. Quinacrine, like many of the other inducers of this pea system,[2–5] has been shown to intercalate[6] into DNA *in vitro* and attach to chromatin[7–9] *in vivo* with predictable specificity. Since all of the structurally defined inducers of PAL and pisatin reported previously[2–5,10–12] have the potential to change the conformation of DNA, the control of this response was proposed[2] to occur at the transcription level. (That is, the DNA becomes more transcribable either by dissociation from a repressor component or by assuming a more desirable conformation for transcription.)

This paper compares the PAL and pisatin induction† potential of chlorpromazine and a series of both patented and experimental phenothiazine derivatives.

MATERIALS AND METHODS

Induction treatments. Immature Alaska pea (*Pisum sativum L.*) pods (less than 2 cm long) were harvested from a greenhouse (10.00 a.m.) while still enclosed in the blossom,

* Scientific paper No. 3610, College of Agriculture, Washington State University, Project 1844.

† The term "inducer" in this paper will refer to an agent which can cause an increase in extractable PAL and pisatin.

to minimize microbial contamination. The pods (1 g/treatment) were split immediately and placed in sterile petri dishes (60 mm dia.). The inducer compounds were dissolved in 1·0 ml H_2O and were sprayed uniformly over the exposed endocarps of the pea pods. The plate then was placed in the dark for an 18-hr incubation period. Inhibitor solutions (0·2 ml), when applied, were mixed with the aqueous solution in the petri plate to bring the final concentration of the inhibitors, cycloheximide and 6-methyl purine, to 0·01 and 0·5 mg/ml respectively. The substituted phenothiazine derivatives 1–10 were obtained from the corresponding pharmaceutical manufacturers. Compounds 14, 15 and 16 were synthesized according to the method of Sunagawa and Ichii.[13] The synthesis of compounds 12, 13 and 17 will be reported elsewhere.

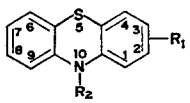
Phenylalanine ammonia lyase extraction and assay. One gram tissue was homogenized in a mortar with 3 ml of a 0·05 M borate buffer (0·5% sodium ascorbate) at pH 8·8, 1 g glass beads and 0·1 g Polyclar. This and subsequent extractive operations were carried out at 2°. The homogenate was filtered through four layers of cheesecloth and centrifuged at 20,000 *g* for 10 min. The supernatant was assayed immediately according to the procedure of Koukol and Conn,[14] revised as follows: the reaction mixture contained 1·5 ml of enzyme homogenate, 20 μmoles L-phenylalanine ($2{\cdot}8 \times 10^5$ dis./min L-[U-^{14}C]phenylalanine) and 200 μmoles of borate buffer, pH 8·8, in a final volume of 2·7 ml. The mixture was incubated for 2 hr at 37°. PAL activity was measured as millimicromoles of [^{14}C]cinnamic acid- produced per gram of fresh tissue per hour. The PAL reaction was linear for at least 3 hr and more than 80 per cent of the enzyme activity was recoverable after storage overnight at 2°.

Extraction and quantitation of pisatin. Pisatin was isolated as described previously.[2] An ethanol extract of pod tissue was taken to dryness and further re-extracted with hexane or petroleum ether. The residue of the hexane extract was separated on Silica gel thin-layer plates. Pisatin was detected on a Silica thin-layer plate by converting it to anhydropisatin in HCl fumes. Anhydropisatin fluoresces under long-wave ultraviolet light. Pisatin was quantitated on the basis of its absorbance at 309 nm.

Rate of synthesis of soluble proteins in pods treated with chlorpromazine sulfoxide. Dual label experiments were used to compare the rates of protein synthesis in induced and non-induced tissues. One 3-g sample of pods was labeled with L-[U-^{14}C]leucine (sp. act., 254 mc/m-mole) 10 hr after inducer application. A second water-treated sample was labeled simultaneously with L-[4,5-^{3}H]leucine (sp. act., 55 c/m-mole). After a 30-min pulse label, the samples were combined and extracted in a mortar containing 10 ml of extraction buffer.

A dual label control experiment utilizing the same harvest of pea pods was performed as described above except that both samples were water-treated. This control experiment was used as a reference and to correct for any isotope effect and differential quenching of samples in the liquid scintillation counter. Each homogenate was centrifuged 10 min at 27,000 *g*. The supernatant solution was adjusted to 80 per cent saturation with ammonia sulfate and centrifuged at 27,000 *g*. The pellet was redissolved in 0·05 M potassium phosphate buffer, pH 7·6, and dialyzed against three changes of the phosphate buffer. The dialyzed protein sample was separated on a Sephadex G-200 column ($110 \times 2{\cdot}5$ cm) and 3-ml fractions were collected. Aliquots (0·7 ml) of each fraction were added to 10 ml of a toluene–Triton X-100 scintillation fluid.[15] The samples were counted in a liquid scintillation counter. Optical density readings (280 nm) were recorded for each sample.

TABLE 1. INDUCTION OF PAL AND PISATIN BY PHENOTHIAZINE DERIVATIVES



Compound	R_1	R_2	Concn. applied* (mg/ml)	PAL activity† (% of H_2O-induced control)	Pisatin‡ (μg/g tissue)
1. Trifluoperazine	2-CF_3	$(CH_2)_3$-N N-CH_3	1·0	320	56
2. Fluphenazine	2-CF_3	$(CH_2)_3$ N $N(CH_2)_2OH$	1·0	249	71
3. Triflupromazine	2-CF_3	$(CH_2)_3$-$N(CH_3)_2$	1·0	550	81
			0·1	251	
4. Prochlorperazine	2-Cl	$(CH_2)_3$-N N-CH_3	1·0	323	33
5. Perphenazine	2-Cl	$(CH_2)_3$ N N-$(CH_2)_2OH$	1·0	532	73
			0·1	260	
6. Chlorpromazine	2-Cl	$(CH_2)_3$-$N(CH_3)_2$	1·0	833	69
7. Chlorpromazine sulfoxide			1·0	1108	96
8. Promethazine	H	CH_2 $CH(CH_3)$-$N(CH_3)_2$	1·0	602	40
			0·1	173	
9. Promazine	H	$(CH_2)_3$ $N(CH_3)_2$	1·0	638	85
			0·1	440	
10. Mepazine	H	CH_2–N, N-CH_3	1·0	384	41
			0·1	179	
11. Thioridiazine	2-SCH_3	$(CH_2)_2$–N, N-CH_3	1·0	404	85
			0·1	208	
12. 10-(1-methyl-1,2,5,6-tetrahydronicotinyl) phenothiazine			1·0	1099	95
13. *N*-(1-methyl-1,2,5,6-tetrahydronicotinyl)-*N*,*N*-diphenylamine			1·0	648	136
H_2O				100	ND§

* The concentrations indicated are the optimal stimulatory concentrations within the range 1·0 to 1×10^{-3} mg/ml.

† PAL activity was assayed 18 hr after inducer application and is expressed as millimicromoles of cinnamic acid per gram of tissue per hour at 39°. The control tissue averaged 110 mμmoles. Variation of PAL activity in the control tissue was slight within a given harvest of pods, but ranged from 46 to 190 mμmoles among harvests.

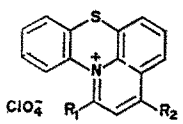
‡ Pisatin was extracted from pods 24 hr after inducer application.

§ ND = nondetectable.

RESULTS

All of the phenothiazine-type drugs 1–13 in Table 1 induce PAL and pisatin formation. The concentrations of inducer necessary for induction typically are in excess of 0·1 mg/ml. These concentrations are substantial when compared to the dosages used in animal systems, but are understandable in view of the generally low permeability of pea tissue to certain organic compounds.[4] In general, the compounds with an aliphatic side chain are superior to those with the piperazine group in inducing both PAL and pisatin. Surprisingly, the sulfoxide of chlorpromazine is the strongest inducer.

TABLE 2. INDUCTION OF PAL AND PISATIN BY PYRIDO [3,2,1-kl] PHENOTHIAZINIUM SALTS



Compound No.	R_1	R_2	Concn. of inducer* (mg/ml)	PAL activity† (% of H_2O-induced control)	Pisatin‡ (μg/g tissue)
14	H	CH_3	1·0	184	122
			0·1	536	
15	H	C_2H_5	1·0	243	38
16	H	C_6H_5	1·0	141	119
17	CH_3	C_6H_5	1·0	559	150
H_2O				100	ND§

* The concentrations indicated are the optimal stimulatory concentrations within the range 1·0 to 1 × 10^{-3} mg/ml.

† PAL activity was assayed 18 hr after inducer application and is expressed as millimicromoles of cinnamic acid per gram of tissue per hour at 39°. The control tissue averaged 110 mμmoles.

‡ Pisatin was extracted from pods 24 hr after inducer application.

§ ND = nondetectable.

The modification of N-10 substitutions to eliminate the basic nitrogen function, as in the salts 14–17 (Table 2), can be detrimental to the induction potential. Compound 16 only slightly increases PAL activity; however, it effectively induces pisatin production. Compound 15 has a low induction potential for both responses.

Chlorpromazine sulfoxide-induced increases in PAL and pisatin depend on RNA and protein synthesis (Table 3). Cycloheximide, when added within 3 hr after inducer application, effectively reduces the maximal induction of PAL. 6-Methylpurine, an inhibitor of all RNA synthesis in plants,[16] significantly obstructs the induced increase in PAL if applied within 1 hr after the application of inducer. In pod tissue, 90 per cent of protein synthesis is inhibited by cycloheximide (0·01 mg/ml) within 1–2 hr after application, and 6-methylpurine (0·5–0·1 mg/ml) inhibits 52–63 per cent of the RNA synthesis within 1 hr after application.

Since the 10-fold increases in PAL activity induced by chlorpromazine sulfoxide apparently require the synthesis of new protein, the effect of this drug on the synthesis of other soluble proteins in pea tissue was examined. Figure 1 demonstrates the

TABLE 3. EFFECT OF 6-METHYLPURINE AND CYCLOHEXIMIDE ON CHLORPROMAZINE SULFOXIDE-INDUCED INCREASES IN PAL

Inducer	Time lapse prior to application of inhibitor (hr)	PAL activity* (% of H_2O-induced control)
H_2O		100
Chlorpromazine sulfoxide†		963
	6-Methylpurine (0·5 mg/ml)	
Chlorpromazine sulfoxide	zero time	151
Chlorpromazine sulfoxide	+ 1	123
Chlorpromazine sulfoxide	+ 3	207
Chlorpromazine sulfoxide	+ 6	293
Chlorpromazine sulfoxide	+ 9	394
	Cycloheximide (0·01 mg/ml)	
Chlorpromazine sulfoxide	zero	123
Chlorpromazine sulfoxide	+ 1	134
Chlorpromazine sulfoxide	+ 3	151
Chlorpromazine sulfoxide	+ 6	381
Chlorpromazine sulfoxide	+ 9	443

* PAL activity was assayed 18 hr after inducer application (see Methods).
† The dose of chlorpromazine sulfoxide was 1 mg/ml in each case.

differential synthesis of proteins fractionated on a Sephadex G-200 column. Significant increases in net rate of protein synthesis occurred in most of the soluble protein fractions, including the fractions containing PAL activity.

DISCUSSION

The drug chlorpromazine is well known for its tranquilizing effects on man and has been shown to have several effects on bacteria and protozoa. These latter effects include inhibition of RNA synthesis,[17] bacteriostasis,[18,19] cessation of mobility,[20] spheroplast induction,[18] cell lysis[21] and enhancement of infectivity.[22]

The results reported herein point out the dramatic changes in enzyme activity which can be induced by optimal concentrations of chlorpromazine and other phenothiazine derivatives in cells of higher organisms.

All of the phenothiazine compounds included in this study were potent inducers of both PAL and pisatin, with the exception of those with a ring structure (e.g. piperazine) in the side chain.

The induction of PAL by chlorpromazine sulfoxide, which depends on RNA and protein synthesis (Table 3),[15] apparently is accompanied by the induced synthesis of many proteins besides those responsible for increasing PAL activity (Fig. 1).

The relative ability of the phenothiazine compounds to penetrate the pea cell or to associate with cellular DNA or to do both, remains to be assessed. However, the similarity of the basic side chain of chlorpromazine to the side chain of quinacrine, chloroquine and spermine suggests that this compound may also be capable of ionic binding with the phosphate groups of DNA.[23–26]

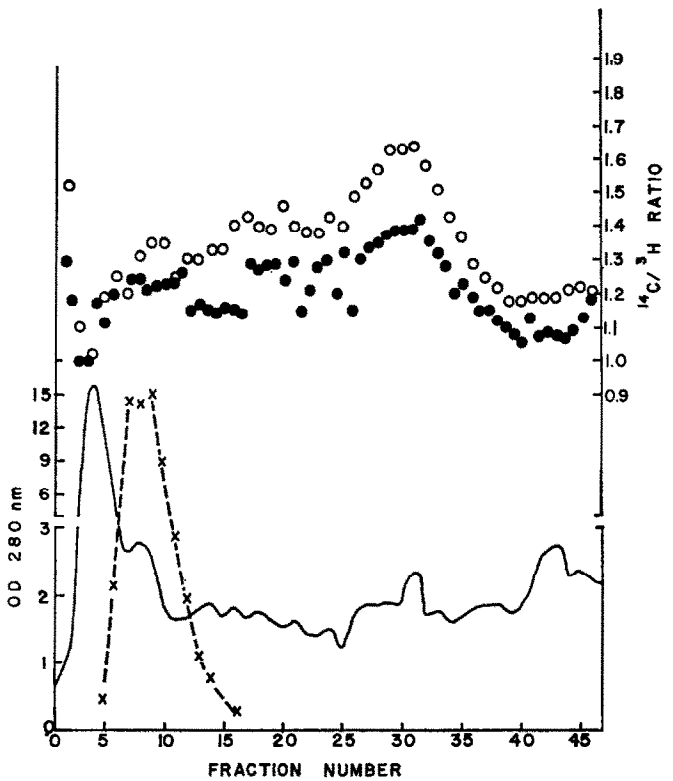


FIG. 1. Relative rate of incorporation of radioactively labeled leucine into soluble proteins of pea pods treated with chlorpromazine sulfoxide (1 mg/ml) versus incorporation into water-treated tissue. One 3-g sample of chlorpromazine sulfoxide-treated pods was labeled with 3 μc L-[H-^{14}C]leucine 9 hr subsequent to drug application. A second sample of water-treated pods was simultaneously labeled with 30 μc L-[4,5-^{3}H]leucine. The two samples were combined and the soluble proteins extracted after a 45-min pulse. The protein was concentrated, dialyzed and subsequently fractionated on a Sephadex G-200 as described previously.[15] The ratios (corrected for counting efficiency and relative counts administered) of [^{14}C]leucine to [^{3}H]leucine in these experiments are indicated by the open circles. The closed circles indicate the ratios of dual labeled control experiments obtained by pulse labeling two water-treated samples (pods were from the same harvest). The solid line indicates optical density at 280 nm. The $\times$'s indicate the fractions containing PAL activity.

We have demonstrated that phenothiazine derivatives have at least one mode of action in common with many DNA intercalating compounds; that is, these compounds increase the activity of one enzyme and the synthesis of various proteins in the cells of a higher organism. This general mode of drug action may relate to the effects or side effects (or both) of many of these compounds in mammalian systems. These simple pea assays may prove to be useful in predicting which of the experimental phenothiazine derivatives possess medicinal promise.

REFERENCES

1. D. A. HUSSAR, *Am. J. Pharm.* **142,** 65 (1970).
2. M. E. SCHWOCHAU and L. A. HADWIGER, *Archs Biochem. Biophys.* **134,** 34 (1969).
3. L. A. HADWIGER and M. E. SCHWOCHAU, *Biochem. biophys. Res. Commun.* **38,** 683 (1970).
4. L. A. HADWIGER and M. E. SCHWOCHAU, *Pl. Physiol. Lancaster* **47,** 375 (1971).

5. M. E. SCHWOCHAU and L. A. HADWIGER, *Archs Biochem. Biophys.* **126,** 731 (1968).
6. L. S. LERMAN, *Proc. natn. Acad. Sci. U.S.A.* **49,** 94 (1963).
7. T. CASPERSSON, L. ZECH, E. J. MODEST, G. E. FOLEY, U. WAGH and E. SIMONSSON, *Expl Cell Res.* **58,** 128 (1969).
8. T. CASPERSSON, L. ZECH, E. J. MODEST, G. E. FOLEY, U. WAGH and E. SIMONSSON, *Expl Cell Res.* **58,** 141 (1969).
9. T. CASPERSSON, L. ZECH and E. J. MODEST, *Science N.Y.* **170,** 762 (1970).
10. I. A. M. CRUICKSHANK and D. R. PERRIN, *Aust. J. biol. Sci.* **16,** 111 (1963).
11. S. LOESCH, M. E. SCHWOCHAU and L. A. HADWIGER, *Pl. Physiol. Lancaster* **44,** 36 (1969).
12. M. E. SCHWOCHAU and L. A. HADWIGER, *Induced Mutations in Plants*, p. 83. International Atomic Energy Agency, Vienna (IAEA-SM-121/32) (1969).
13. G. SUNAGAWA and T. ICHII, *J. pharm. Soc. Japan* **79,** 1409 (1959).
14. J. KOUKOL and E. E. CONN, *J. biol. Chem.* **236,** 2692 (1961).
15. M. S. PATTERSON and R. C. GREEN, *Analyt. Chem.* **37,** 854 (1965).
16. J. L. KEY and J. SHANNON, *Pl. Physiol. Lancaster* **39,** 360 (1964).
17. R. SUTRA, *C.r. hebd. Séanc. Acad. Sci., Paris* **272,** 2784 (1966).
18. J. L. BOURDON, *Annls Inst. Pasteur, Paris* **101,** 876 (1961).
19. P. KLUBES, G. TABOR and H. MANDEL, *Pharmacologist* **9,** 193 (1967).
20. C. G. ROGERS, *Can. J. Biochem. Physiol.* **44,** 1493 (1966).
21. R. P. AGARWAL and A. GUHA, *Br. J. Pharmac. Chemother.* **24,** 466 (1965).
22. G. T. HEBERLEIN, *Infec. Immun.* **2,** 468 (1970).
23. R. L. O'BRIEN, J. G. OLENICK and F. E. HAHN, *Proc. natn. Acad. Sci. U.S.A.* **55,** 1511 (1966).
24. J. CIAK and F. E. HAHN, *Science, N.Y.* **156,** 655 (1967).
25. J. L. ALLISON, R. L. O'BRIEN and F. E. HAHN, *Science, N.Y.* **149,** 1111 (1965).
26. H. TABOR, *Biochemistry, N.Y.* **1,** 496 (1962).