Thionins: Plant Peptides that Modify Membrane Permeability in Cultured Mammalian Cells

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Thionins, which are high-sulphur polypeptides present in the endosperm of wheat and related species, have been found to prevent growth and to inhibit macromolecular synthesis in cultured mammalian cells. Baby hamster kidney (BHK) cells were markedly more sensitive to thionins than the other cell lines tested (monkey CV1, mouse L, human HeLa). A thionin concentration of 5 μg/ml (1 μM) completely blocked translation in BHK cells. It was later found that omission of both calcium and magnesium ions from the medium strongly enhanced the inhibitory effects of thionins (BHK cells, 80% inhibition, 0.5 μg/ml). Several lines of evidence indicate that thionins might act at the membrane level. Indeed, both the $^{86}Rb^+$ content and the nucleotide pool of BHK cells were drastically decreased at thionin concentrations that inhibited translation. In addition, thionin concentrations that did not affect macromolecular synthesis in these cells, allowed inhibition of translation by antibiotics, such as hygromycin B, that are not able to cross the cell plasma membrane by themselves. Our results suggest that the inhibition of protein, RNA and DNA synthesis in BHK cells might be a consequence of membrane leakiness induced by thionin treatment. In this respect, particularly striking was the parallelism found between $^{86}Rb^+$ leakage and inhibition of protein synthesis by treatment with different genetic variants of thionins (α_1 purothionin, α_2 purothionin, β purothionin from wheat; hordothionin from barley), as well as with the viscotoxins, which are homologous polypeptides from the European mistletoe.

The thionins are cystine-rich, basic polypeptides of M_r about 5000, which are quite abundant in the endosperms of many Gramineae [1–5]. Several of them have been sequenced [6–11] and found to be homologous to the viscotoxins from the European mistletoe (*Viscum album* L.) and related species [12]. The thionins are soluble in water and in 90% ethanol, and can be extracted from endosperm as free proteins with dilute acid (25 mM H_2SO_4) or as protein-lipid complexes with petroleum ether [4]. In hexaploid wheat, *Triticum aestivum* L., there are three genetic variants, designated β, α_B and α_D purothionins, which are encoded by genes *purA1*, *purB1* and *purD1*, located in the long arms of chromosomes 1A, 1B and 1D, respectively [4]. They are toxic to laboratory animals [13], insect larvae [14], bacteria and yeast [15–17]. Effects on uterus muscle from the guinea pig [13] and on insect flight muscle [14] have also been reported. Although a crude preparation from wheat endosperm, which probably contained thionins, was found to induce leakage from yeast cells [18] and purified thionins were shown to have the same effect on bacteria [19], little is actually known about the mode of action of thionins at the molecular level.

We report here the modification of membrane permeability and the inhibition of macromolecular synthesis by thionins in cultured mammalian cells.

MATERIALS AND METHODS

Cells

Mouse fibroblasts L929 cells, hamster BHK-21 cells, human HeLa cells and monkey CV1 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% newborn calf serum (Difco) (E4D10 medium).

Protein Synthesis Measurement

Cells were grown in 24-well limbro dishes in 1 ml E4D10 medium. At the indicated times, the medium was replaced by 0.5 ml of methionine-free medium with the indicated ionic characteristics and supplemented with 1% newborn calf serum. The thionins were added as indicated in each experiment and protein synthesis was estimated by addition of 0.1–0.2 μCi [^{35}S]methionine (1000 Ci/mmol, 5.4 mCi/ml, Amersham). After incubation, the medium was removed and the cell monolayer treated with 5% trichloroacetic acid, washed twice with ethanol and dissolved in 0.200 ml 0.1 M NaOH; 0.150 ml were taken to estimate radioactivity in a scintillation spectrometer.

DNA and RNA Synthesis Measurement

DNA synthesis was measured by estimating the incorporation of [*methyl*-^{3}H]thymidine (52 Ci/mmol; 1 mCi/ml) into trichloroacetic-acid-precipitable material as indicated above. RNA synthesis was measured by estimating the incorporation of [5,6-^{3}H]uridine (48 Ci/mmol; 1 mCi/ml) into trichloroacetic-acid-precipitable material.

Measurement of $^{86}Rb^+$ Content

BHK 21 cells grown in 24-well plates as described above were placed in 0.5 ml E4D1 medium. To load the cells with $^{86}Rb^+$, 0.2 μCi $^{86}Rb^+$ (Amersham, 1 mCi/ml) were added and

the cells were incubated for the indicated periods of time and the thionins were added. At the indicated times, the medium was removed and the cells washed three times with 1 ml saline phosphate buffer and 0.5 ml 5% trichloroacetic acid was added to extract the $^{86}Rb^+$ in the cells. 0.250-ml aliquots of this extract were withdrawn and mixed with 1 ml of water and the radioactivity was determined by estimating the Cerenkov radiation in a liquid scintillation spectrometer.

Purification of Toxins

A mixture of the three thionins from hexaploid wheat (α_1, α_2 and β purothionins) was obtained from *Triticum aestivum* L. cv. Aragon 03 by petroleum ether extraction, precipitation with 3 vol. 1 M HCl in ethanol, and preparative electrophoresis in polyacrylamide columns as previously described [17]. The individual genetic variants of purothionin (α_1, α_2 and β) were obtained from a mixture of the three by carboxymethyl-cellulose chromatography (0.3–0.7 M ammonium acetate, pH 5.2) as described by Jones and Mak [8]. The thionin from barley, hordothionin, was obtained from *Hordeum vulgare* L. cv. Zephyr, by carboxymethyl-cellulose chromatography of the 1 M HCl/ethanol precipitate. Purity of each variant was checked by two-dimensional starch-gel electrophoresis (0.015 M aluminium lactate/lactic acid buffer, pH 3.2, containing 3 M urea, in the first dimension, and 0.02 M sodium acetate/acetic acid buffer, pH 5.2 in the second dimension) and by amino acid analysis.

A partially purified mixture of viscotoxins was obtained from samples of European mistletoe (*Viscum album* L.) collected at the Balsain Forest near Madrid. These were extracted with 2.5 vol. of 3% acetic acid in a Sorvall Omnimixer at 4 °C. The homogenate was centrifuged (16000 × *g*, 30 min) and the supernatant freeze-dried. To eliminate pigments and some other non-protein components the freeze-dried material was dissolved in 60% ethanol, 20% acetic acid, and the highly pigmented insoluble fraction discarded. The solution was brought up to 72% ethanol, 1.25 M HCl, and the precipitate discarded. A fraction representing about 80% of the original protein was precipitated overnight with 90% ethanol at 4 °C. The precipitate was dissolved in 3% acetic acid and fractionated in a Sephadex G-100 column. The fraction with M_r under 10000 was further fractionated in a carboxymethyl-cellulose column (0.1–0.5 M ammonium acetate, pH 5.2). Fractions showing cytopathic effects were pooled and repeatedly freeze-dried.

RESULTS

Action of Thionins on Macromolecular Synthesis in Cultured Mammalian Cells

In an attempt to clarify the molecular mechanism of action of thionins we started by testing the inhibition exerted by $\alpha_1\alpha_2\beta$ purothionin on translation in several mammalian cell lines. Fig. 1 shows that baby hamster kidney (BHK) cells were, amongst the cell lines tested, the most susceptible to the inhibitory action of thionins. This selectivity does not seem to be the consequence of a greater fragility or instability of the BHK cell membrane towards membrane-active agents, because it was not found for other agents, such as nigericin or melittin (unpublished observations).

To optimize the inhibition of translation by thionins in BHK cells, we modified several conditions in the culture medium, including the divalent cation concentration. Fig. 2

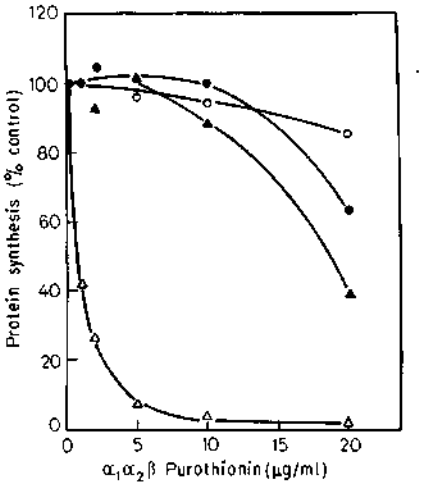

Fig. 1. *Action of thionins on translation in different mammalian cell lines.* The concentration of thionin indicated in the figure was added at zero time and the cells were incubated for 4 h at 37 °C. Protein synthesis was estimated by incubating with [^{35}S]methionine for an additional 1 h at 37 °C as indicated in Materials and Methods. (●——●) Mouse L_{929} cells (100% control represents 1823 counts/min); (○——○) human HeLa cells (100%: 3314 counts/min); (▲——▲) monkey CV1 cells (100%: 1229 counts/min); (△——△) hamster BHK cells (100%: 2363 counts/min)

indicates that the concentration of calcium ions in the medium strongly influences the inhibitory capacity of thionins. A complete reversion of this inhibition is obtained in the presence of 5 mM $CaCl_2$, whereas maximal inhibition occurs when both calcium and magnesium ions are absent from the culture medium. Under these conditions, a concentration of 0.5 μg/ml (0.1 μM) $\alpha_1\alpha_2\beta$ purothionin inhibited protein synthesis almost by 80% after 4 h of treatment.

The time course of the inhibition of translation by $\alpha_1\alpha_2\beta$ purothionin is shown in Fig. 3. A lag of about 2–3 h is needed before maximal effect of thionins is attained. This time period is probably required for the toxin to interact with the cellular surface and to bring about the inhibition of translation.

To know how specific the inhibition by thionins on protein synthesis was, we measured DNA and RNA synthesis in BHK cells under different thionin concentrations. Fig. 4 indicates that all three activities tested in BHK cells, DNA, RNA and protein synthesis, were inhibited by $\alpha_1\alpha_2\beta$ purothionin, though to a different extent, protein synthesis being the most sensitive to toxin treatment.

Modification of Membrane Permeability by Thionins

To investigate whether the inhibition of macromolecular synthesis by thionins could be a consequence of membrane leakiness induced by the toxin, cells were loaded with the radioactive potassium analog $^{86}Rb^+$ and assayed for leakiness to this ion in the presence of $\alpha_1\alpha_2\beta$ purothionin. Fig. 5 shows that indeed thionins caused a clear and immediate leakage of $^{86}Rb^+$ ions from the cells and both the extent and the speed of this leakage were influenced by the concentration of $\alpha_1\alpha_2\beta$ purothionin present in the culture medium. The leakiness to other low-molecular-weight compounds present in the cell cytoplasm, like uridine (or its nucleotide derivatives) is also apparent after thionin treatment (Fig. 6). These results are in good agreement with previous reports showing that wheat extracts permeabilized microorganisms to ultraviolet-absorbing substances [18, 19]. In addition, the capacity

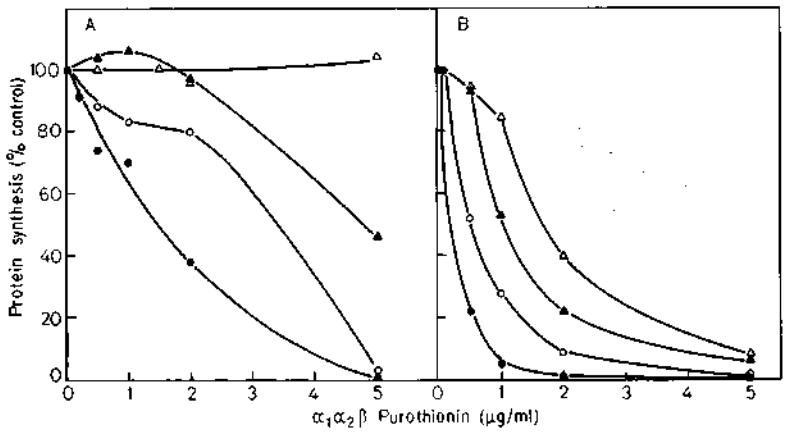

Fig. 2. *Effect of divalent cations on the inhibition of translation in BHK cells by thionins.* Cells were grown in Linbro dishes as indicated in Materials and Methods. The growth medium was replaced by medium with different concentrations of calcium ions (A) or medium without calcium and different concentrations of magnesium ions (B). (A) (●——●) No calcium added (100%: 30471 counts/min); (○——○) 0.5 mM Ca^{2+} (100%: 27008 counts/min); (▲——▲) 1 mM Ca^{2+} (100%: 25160 counts/min); (△——△) 5 mM Ca^{2+} (100%: 25175 counts/min). (B). (●——●) No magnesium added (100%: 28100 counts/min); (○——○) 0.2 mM Mg^{2+} (100%: 29281 counts/min); (▲——▲) 0.8 mM Mg^{2+} (100%: 23364 counts/min); (△——△) 2 mM Mg^{2+} (100%: 26327 counts/min). protein synthesis was estimated as indicated in Fig. 1

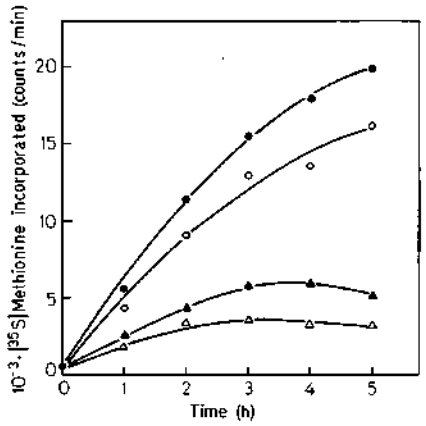

Fig. 3. *Time course of translation in BHK cells: effect of different concentrations of thionins.* Protein synthesis was estimated as in Fig. 1, in culture medium without calcium and magnesium ions (●——●) Control; (○——○) 0.2 μg/ml $\alpha_1\alpha_2\beta$ purothionin (▲——▲) 0.5 μg/ml $\alpha_1\alpha_2\beta$ purothionin; (△——△) 1 μg/ml $\alpha_1\alpha_2\beta$ purothionin

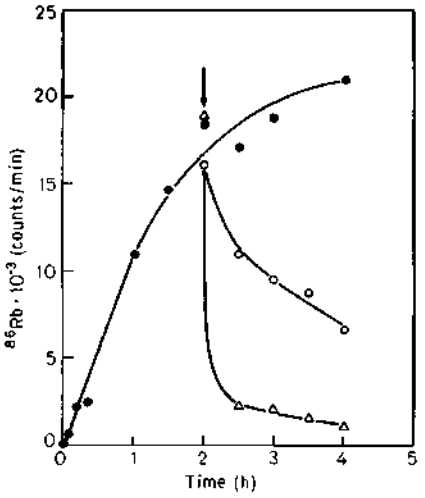

Fig. 5. *Effect of thionins on $^{86}Rb^+$ content in BHK cells.* $^{86}Rb^+$ was estimated as indicated in Materials and Methods. The arrow indicates the time when the thionins were added. (●——●) Control; (○——○) 1 μg/ml $\alpha_1\alpha_2\beta$ purothionin; (△——△) 5 μg/ml $\alpha_1\alpha_2\beta$ purothionin

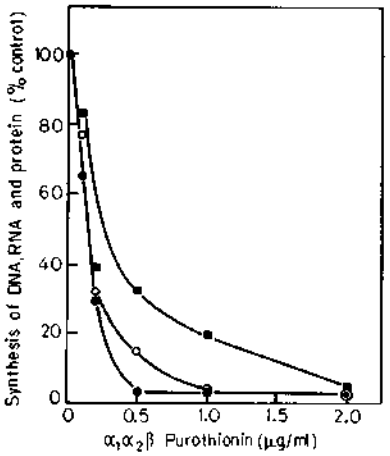

Fig. 4. *Effect of thionins on macromolecular synthesis in BHK cells.* The concentrations of thionins indicated in the figure were added to BHK cells in medium without calcium and magnesium. After 4 h of incubation with $\alpha_1\alpha_2\beta$ purothionin, DNA synthesis (■——■) (100%: 67586 counts/min), RNA synthesis (○——○) (100%: 14287 counts/min) and protein synthesis (●——●) (100%: 39934 counts/min) were estimated as indicated in Materials and Methods

of BHK cells to exclude some antibiotics like hygromycin B, that do not cross the membrane of normal cells was also tested in the presence of different concentrations of the toxin (Fig. 7). Thionins modified the membrane permeability towards hygromycin B at lower concentrations than those necessary to block protein synthesis and to induce $^{86}Rb^+$ leakage. It should be noted that in this experiment, the standard concentration of divalent cations was present in the culture medium, and hence a higher concentration of thionins was necessary to block protein synthesis.

Comparison of Activities of Several Genetic Variants of Thionins

To test the parallelism between the inhibition of translation and the induction of $^{86}Rb^+$ leakage further, as well as to compare the activities of different genetic variants of thionins, the experiments summarized in Fig. 8 were carried out. The purified thionins α_1 purothionin, α_2 purothionin and β purothionin from wheat, hordothionin from barley, and

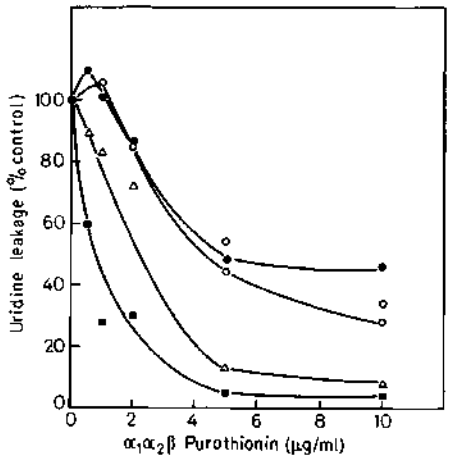

Fig. 6. *Effect of thionins on the uridine pool.* BHK cells grown in Linbro dishes were changed to a medium without calcium and magnesium. 1 μCi [5,6-^{3}H]uridine (48 Ci/mmol; 1 mCi/ml) was added per well and incubated 3 h at 37 °C in the presence of 10 μg/ml actinomycin D. After this incubation the indicated concentration of $\alpha_1\alpha_2\beta$ purothionin was added and the incubation pursued for 1 h (●——●) (100%: 11942 counts/min), 2 h (○——○) (100%: 8641 counts/min) or 4 h (△——△) (100%: 7923 counts/min); (■——■) 4-h incubation in the presence of purified β thionin (100%: 8437 counts/min). After this time the trichloroacetic-acid-soluble material present in the BHK cell monolayer was measured

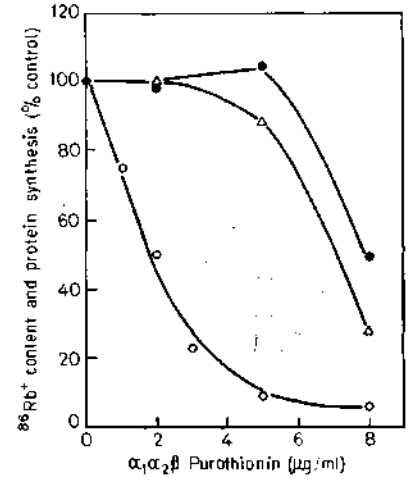

Fig. 7. *Effect of thionins on translation, $^{86}Rb^+$ content and permeability to hygromycin B.* The effect of different concentrations of thionins on $^{86}Rb^+$ content (△——△) (100%: 3552 counts/min) and protein synthesis in the absence (●——●) (100%: 5209 counts/min) or in the presence of 2 mM hygromycin B (○——○) (100%: 6543 counts/min) was estimated after a 4-h incubation in normal medium. Protein synthesis and $^{86}Rb^+$ content were measured as indicated in Materials and Methods

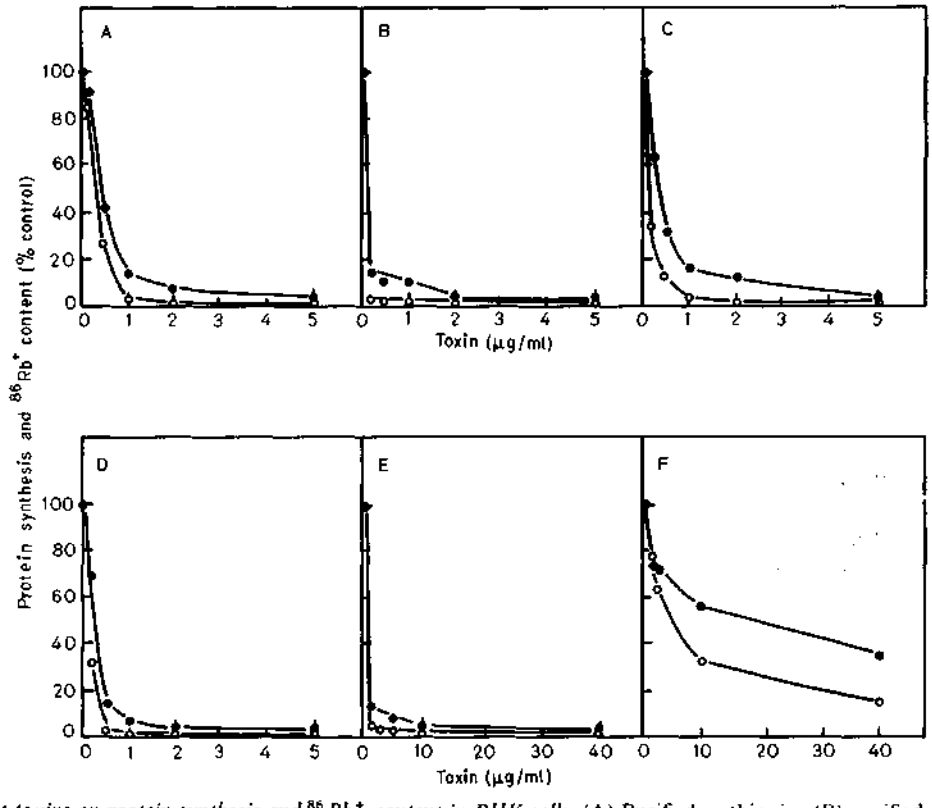

Fig. 8. *Effect of different toxins on protein synthesis and $^{86}Rb^+$ content in BHK cells.* (A) Purified α_2 thionin; (B) purified β thionin; (C) $\alpha_1\alpha_2\beta$ purothionin mixture; (D) purified α_1 thionin; (E) hordothionin from barley; (F) viscotoxins from european mistletoe. BHK cells were incubated in the presence of the toxins as indicated above for 4 h in medium without calcium and magnesium. Protein synthesis (○——○) and $^{86}Rb^+$ content (●——●) were estimated as indicated in Materials and Methods

a partially purified mixture of viscotoxins, which are peptides from mistletoe homologous to the thionins, were all very active in promoting both the inhibition of translation and $^{86}Rb^+$ leakage in BHK cells (Fig. 8). The parallelism observed in the variation of the two parameters is consistent with the idea that an alteration of the potassium content in toxin-treated cells might be responsible for the observed inhibition of protein synthesis.

DISCUSSION

There is an increasing number of natural compounds that have been found to be active on the cell membrane [20–22]. These compounds are normally cytocidal as they produce profound and irreversible damage to the cell, in a similar manner to that observed for the series of related polypeptides studied here.

The idea that thionins interact with the membrane is supported by our finding that thionin treatment of BHK cells induces a rapid leakage of ions and of low-molecular-weight compounds from those cells. Moreover, entrance of low-molecular-weight compounds, such as the translation inhibitor hygromicin B [23], is also facilitated by thionin at concentrations much lower than those required to induce leakage; similar findings have been reported for the ionophore nigericin [24]. These observations are also in accordance with the known physical characteristics of thionins, as it has been reported that they are able to strongly bind polar lipids phosphatidylcholine, phosphatidylethanolamine and digalactosyl diglyceride, and to become soluble in chloroform and in petroleum ether in association which these lipids [25]. The ability of divalent cations to interfere with thionin action could then be explained in terms of the cations preventing the interaction of the positively charged thionins with membrane phospholipids.

The inhibition by thionins of macromolecular synthesis *in vivo*, which had not been previously reported, is probably a consenquence of the alteration of the ionic composition of the cellular cytoplasm brought about by the thionin-induced permeabilization of the cell membrane. Nevertheless, direct inhibition by thionins of specific steps of DNA, RNA and protein synthesis is not excluded by the experiments reported here.

Thionins have a broad spectrum of action, as they are active on both gram-positive and gram-negative bacteria, as well as on eukaryotes, including yeast and animal cells [13–15, 17]. However there are significant differences in sensitivity among the cultured mammalian cell lines tested, hamster BHK cells being particularly sensitive. The fact that other membrane-active compounds, such as nigericin or melittin, do not show the same specificity suggests a different molecular target for the action of thionins. The molecular basis of the observed selectivity still remains unknown.

Thionins are widespread among the endosperms of many Gramineae, where they are quite abundant. Their physiological function is as yet unknown, although they could certainly have the role of protecting the starchy endosperm against microbial infection during germination.

The apparent lack of toxicity to man, derived from thionins present in wheat flour, which is the main single source of protein for a large fraction of the world population, is probably due either to proteolytic degradation in the digestive tract or to inactivation during backing.

The viscotoxins are homologous to the thionins, having in common more than half of their amino acid sequence, although they have three instead of four disulphide bridges [7]. They occur in many species of the family Loranthaceae, where they present considerable genetic variability [12]. The viscotoxins are also toxic to laboratory animals on parentereal administration and, in sublethal doses, produce hypotension, bradycardia and a negative inotropic effect on heart muscle [12]. Our present results further indicate a common mechanism of action for thionins and viscotoxins, in spite of the considerable evolutionary divergence from their common origin shown by both series of polypeptides.

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REFERENCES

1. Balls, A. K. & Hale, W. S. (1942) *Cereal Chem. 19*, 279–288.
2. Carbonero, P. & García-Olmedo, F. (1969) *Experientia (Basel) 25*, 1110.
3. Redman, G. D. & Fisher, N. (1969) *J. Sci. Food Agr. 20*, 427–432.
4. Fernández de Caleya, R., Hernández-Lucas, C., Carbonero, P. & Garcia-Olmedo, F. (1974) *Genetics, 83*, 687–699.
5. Hernández-Lucas, C., Carbonero, P. & García-Olmedo, F. (1978) *J. Agric. Food Chem. 26*, 794–796.
6. Othani, S., Okada, T., Kagamiyama, H. & Yoshizumi, H. (1975) *Agric. Biol. Chem. 39*, 2269–2270.
7. Mak, A. S. & Jones, B. L. (1976) *Can. J. Biochem. 54*, 835–842.
8. Jones, B. L. & Mak, A. S. (1977) *Cereal Chem. 54*, 511–523.
9. Othani, S., Okada, T., Yoshizumi, H. & Kagamiyama, H. (1977) *J. Biochem. (Tokyo) 82*, 753–767.
10. Hase, T., Matsubara, H. & Yoshizumi, H. (1978) *J. Biochem. (Tokyo) 83*, 1671–1678.
11. Ozaki, Y., Wada, K., Hase, T., Matsubara, H., Nakanishi, T. & Yoshizumi, H. (1980) *J. Biochem. (Tokyo) 87*, 549–555.
12. Samuelsson, G. (1973) *Syst. Zool. 22*, 566–570.
13. Coulson, E. J., Harris, T. H. & Axelrod, B. (1942) *Cereal Chem. 19*, 301–307.
14. Kramer, K. J., Klassen, L. W., Jones, B. L., Speirs, R. D. & Kammer, A. E. (1979) *Toxicol Appl. Pharmacol. 48*, 179–183.
15. Stuart, L. S. & Harris, T. H. (1942) *Cereal Chem. 19*, 288–300.
16. Fernández de Caleya, R., González, B., García-Olmedo, F. & Carbonero, P. (1972) *Appl. Microbiol. 23*, 998–1000.
17. Hernández-Lucas, C., Fernández de Caleya, R., Carbonero, P. (1974) *Appl. Microbiol. 28*, 165–168.
18. Okada, T. & Yoshizumi, H. (1973) *Agr. Biol. Chem. 37*, 2289–2294.
19. Fernández de Caleya, R. (1973) Dr. Ing. Agr. Thesis, Universidad Politécnica de Madrid.
20. McLaughlin, S. & Eisenberg, M. (1975) *Annu. Rev. Biophys. Bioeng. 4*, 335–366.
21. Pressman, B. C. (1976) *Annu. Rev. Biochem. 45*, 501–530.
22. Ovchinnikov, Y. A. (1979) *Eur. J. Biochem. 94*, 321–336.
23. Vázquez, D. (1979) in *Molecular Biology, Biochemistry and Biophysics*, vol. 30, Springer-Verlag, Berlin.
24. Alonso, M. A. & Carrasco, L. (1980) *Eur. J. Biochem. 109*, 535–540.
25. Hernández-Lucas, C., Fernández de Caleya, R., Carbonero, P. & Garcia-Olmedo, F. (1977) *J. Agric. Food Chem. 25*, 1287–1289.

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