Studies on the Proteins from the Seeds of *Croton tiglium* and of *Jatropha curcas*

TOXIC PROPERTIES AND INHIBITION OF PROTEIN SYNTHESIS *IN VITRO*

By FIORENZO STIRPE,* ANNALISA PESSION-BRIZZI,* ENZO LORENZONI,*
PAOLA STROCCHI,† LUCIO MONTANARO* and SIMONETTA SPERTI*

*Istituto di Patologia Generale and †Istituto di Farmacologia dell'Università di Bologna,
40126 Bologna, Italy

(Received 5 June 1975)

1. Proteins extracted from the seeds of the Euphorbiaceae *Croton tiglium* and *Jatropha curcas* were separated into three major peaks (I, II and III) by Sephadex chromatography.
2. The crude protein from both seeds and peaks I and II from *Croton* and peak I from *Jatropha* were toxic to mice, to different extents.
3. The crude protein and peak I and peak II from both seeds, inhibited protein synthesis by a reticulocyte lysate; maximum inhibition was exerted by peak II from both seeds. None of these preparations affected protein synthesis *in vitro* by Ehrlich ascites cells.

The toxicity of the seeds of the Euphorbiaceae *Croton tiglium* has been known for a long time, and has been attributed in part to their irritating oil and in part to a protein constituent, 'crotonin', sometimes indicated as consisting of a 'crotonglobulin' and a 'crotonalbumin' (Kraemer, 1915; Wehmer, 1931). In the early literature crotonin was often associated with ricin and abrin, the toxic proteins from *Ricinus communis* and *Abrus precatorius* respectively (Jacoby, 1924). According to Nicolle & Cesari (1913) the lesions caused in the rabbit by ricin, abrin and crotonin are identical, and resemble those brought about by diphtheria toxin. The less known 'curcin', a toxic protein from the seeds of the Euphorbiaceae *Jatropha curcas* has also been included in the same group of toxins, being considered as similar to ricin (Wehmer, 1931; Schwarting, 1963).

More recently it has been observed that ricin and abrin are powerful inhibitors of protein synthesis in whole cells (Lin *et al.*, 1971) and in cell-free systems (Olsnes & Pihl, 1972). They act in an identical fashion by damaging the 60S ribosomal subunit, making ribosomes unable to form a stable complex with elongation factor 2 (Montanaro *et al.*, 1973, 1975; Sperti *et al.*, 1973; Carrasco *et al.*, 1975), whereas diphtheria toxin affects translocation by inactivating elongation factor 2 (see review by Pappenheimer & Gill, 1973).

The aim of the present experiments was to ascertain whether crotonin and curcin inhibit protein synthesis, as do the other toxins mentioned above. It was observed that the seeds of *Croton tiglium* and

Jatropha curcas contain proteins that are toxic to animals and inhibit protein synthesis in a cell-free system, but not in whole cells.

Experimental

Materials

Seeds from *Croton tiglium*, originally from Ceylon, were obtained from Dr. C. Sessa S.p.A., Milan, Italy or from Mr. F. G. Celo, Zweibrücken, West Germany; the seeds of *Jatropha curcas*, originally from India, were also supplied by Mr. F. G. Celo. Ehrlich ascites tumours were a kind gift from Dr. F. Spreafico, Milan, Italy, and were transplanted into Swiss mice. L-[¹⁴C]Leucine (specific radioactivity 330-348 mCi/mmol) was from The Radiochemical Centre, Amersham, Bucks., U.K.; poly-L-ornithine hydrobromide (mol.wt. 122000), ATP and bovine serum albumin were from Sigma Chemical Co., St. Louis, MO, U.S.A.; insolubilized trypsin and chymotrypsin (Enzyte) and GTP were from Miles Research Laboratories, Slough, Bucks., U.K.; creatine phosphate and creatine kinase were from Boehringer Mannheim G.m.b.H., Mannheim, West Germany. All other chemicals were of analytical grade.

Toxicity experiments

Toxicity was evaluated in male Swiss mice weighing 25-28 g, kept in individual cages and supplied with food and water *ad libitum*. Samples of the extracts, diluted with 0.9% NaCl, were injected intraperitoneally into groups of four animals per dose.

LD₅₀ was calculated by the probit method (Finney, 1964). The general behavioural and neurological (somatic and vegetative) state of the animals was evaluated daily by the procedure of Irwin (1964, 1968), and the results were scored on a semi-quantitative scale or on a quantal scale (the frequency of animals showing a given symptom). Semi-quantitative values were statistically evaluated by the Student's *t* test, each group being compared with a control group on the same day. Quantal values were compared by using the significance limits for the fourfold table test (Diem & Lentner, 1972).

Protein synthesis in vitro

Protein synthesis was measured with a lysate of rabbit reticulocytes or with Ehrlich ascites cells. A lysate of reticulocytes was prepared as described by Allen & Schweet (1962), and protein synthesis was assayed as described by Olsnes & Pihl (1972). After incubation, samples were withdrawn and added to 1 ml of 0.1 M-KOH. After 30 min at room temperature (22–25°C), samples were decolorized with a drop of H₂O₂; an equal volume of 20% (w/v) trichloroacetic acid was added, and the protein precipitate was collected on glass-fibre filters (Whatman GF/C), which were washed several times with 5% trichloroacetic acid.

Protein synthesis was measured by the method of Refsnes *et al.* (1974) in Ehrlich ascites cells, washed as described by the same authors. Samples were taken and treated as described above.

General

Radioactivity was determined in a Packard Tri-Carb liquid-scintillation spectrometer, with an external standard: filters were put in counting vials with 6 ml of glycol monomethyl ether and 10 ml of scintillation fluid [0.05% 1,4-bis-(5-phenyloxazol-2-yl)benzene and 0.4% 2,5-diphenyloxazole, in toluene]. Counting efficiency was approx. 55%.

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard.

Results

Extraction of seeds

Croton tiglium. Portions (up to 100 g) of decorticated seeds were extracted with 8 × 250 ml of ethyl ether, each time in a Waring blender; ether was removed by filtering, with suction, through filter paper. The resulting powder was allowed to dry in the air, and was then extracted with 1 litre of cold 0.005 M-sodium phosphate buffer, pH 7.2, containing 0.2 M-NaCl/100 g of seeds. The extraction was carried out in a Waring blender operated for 1 min periods at 5 min intervals for 1 h. The mixture was stirred on a magnetic stirrer for 2–3 h, and was then left

overnight in the cold-room. After centrifugation, the supernatant was brought to 100% saturation with solid (NH₄)₂SO₄, added slowly with constant stirring, which was continued for 30–60 min after the addition of the salt. After at least 3 h the protein precipitate was collected by centrifugation at 10000 g for 20 min at 2°C; it was then dissolved in the minimum amount of phosphate/NaCl buffer, and was dialysed for 24–48 h against a continuous flow of the same buffer. At the end of dialysis a brown precipitate was present in the bags, which was removed by centrifugation. Some of the material present in the supernatant tended to precipitate on further cooling, for instance when transferred from the cold-room (4°C) to an ice bucket or to a centrifuge set at 0°C; however, the precipitate redissolved rapidly on increasing the temperature. The brownish supernatant, referred to as crude croton, was loaded on a column (115 cm × 4.3 cm) of Sephadex G-100, previously equilibrated with the phosphate/NaCl buffer. The column was eluted with the same buffer, and 12 ml fractions were collected, the *E*₂₈₀ being recorded. Three peaks were obtained, referred to as I, II and III in the order of elution, with the maximum emerging at fractions 45, 100 and 135–160 respectively. The fractions from each peak were pooled, the protein was precipitated with (NH₄)₂SO₄ (100% satd.), redissolved in phosphate/NaCl buffer and dialysed against the same buffer. The protein from each peak will be referred to as croton I, II and III, according to the peak. The preparations could be stored for months at –25°C, and could be thawed and frozen again several times without appreciable loss of activity.

Attempts to purify further the active fractions by the methods described for the purification of ricin (Nicolson & Blaustein, 1972; Olsnes & Pihl, 1973; Hara *et al.*, 1974) were unsuccessful; in particular, no protein remained attached to Sepharose 4B columns.

Jatropha curcas. The same procedures as described above were followed for the extraction of the seeds, for the preparation of the crude protein (referred to as crude curcin) and for its fractionation on Sephadex, which again gave three peaks, referred to as curcin I, II and III in the order of elution, similar to those obtained from Croton. As compared with Croton, the following differences were observed: (i) the precipitation of material on further cooling was more abundant; (ii) the activity (see below) of curcin II decreased markedly within a few days at –25°C; (iii) the material of peak III could not be precipitated with saturated (NH₄)₂SO₄, and was discarded.

Toxicity

Croton tiglium. Crude croton, croton I and II, but not croton III were toxic to mice. Symptoms did not

begin until 24 h after poisoning, and deaths mostly occurred after 48 h. Animals receiving crotin II died later; at the lowest doses deaths occurred even after 15 days of poisoning. For this reason an acute and a delayed LD₅₀ were calculated at 48 h and at 7 days respectively (Table 1). The LD₅₀ of crude crotin was significantly higher than expected on the basis of the hypothesis of additivity (Finney, 1964); thus at 72 h crude crotin was significantly less toxic than was expected from the toxicity of crotin I and II. Crotin I was more toxic than crotin II, to a statistically significant extent.

The Irwin's tests (Irwin, 1964, 1968) showed significant alterations of behaviour, such as (Irwin's terminology) transfer arousal, decreased locomotor activity and positional passivity, impairment of corneal and pinna reflexes, neurological symptoms, such as loss of grip strength and impairment of righting reflex, and autonomic symptoms, such as palpebral closure and defecation (presence of very dark faeces in and around the anus).

Post-mortem examinations showed alterations of the liver, which was enlarged, often yellow, sometimes with red patches of necrosis. Kidneys were pale. Ascites was present in some animals poisoned with crude crotin, more often in those treated with crotin II.

Jatropha curcas. Crude curcin and curcin I were also toxic to mice, although less than the corresponding preparations from Croton. As compared with crotins, their action was slightly more rapid, symptoms beginning at 12 h and most deaths occurring within 48 h of poisoning. Therefore the acute LD₅₀ was calculated at 48 h (Table 1). Curcin II was not toxic up to 6 mg of protein per mouse.

The behaviour of the animals was similar to that of mice treated with crotins, except for some neurological symptoms (waddling, fine tremors, rocking), which were present especially among animals poisoned with the highest doses of curcin I; some of these animals had convulsions.

Post-mortem examinations showed lesions in the liver, pancreas and spleen, hyperaemia of the intestine, sometimes ascites; the whole picture resembled that of rats poisoned with ricin (Flexner, 1897; Waller *et al.*, 1966; Derenzini *et al.*, 1976).

Effect on protein synthesis

Cell-free system. Incorporation of [¹⁴C]leucine into protein by a lysate of rabbit reticulocytes was inhibited by crude crotin (Fig. 1a). Some inhibition was also exerted with crotin I (Fig. 1b), whereas crotin II had a much higher inhibitory activity (Fig. 1c). Crotin III inhibited significantly only at much higher concentrations (about 100 µg/ml; results not shown).

Similar results were obtained with the preparations from *Jatropha*. Among these, crude curcin was much more inhibitory than crude crotin (Fig. 2a), curcin I was less active (Fig. 2b), and the inhibitory activity of curcin II was no higher than that of the crude protein (Fig. 2c). Since the activity of curcin II decreases rapidly on storage at -25°C, it is possible that some activity had already been lost during chromatography and dialysis. Curcin III was not tested.

All preparations lost their inhibitory activity if they were heated in a boiling-water bath for 5 min. The activity of crotin I and II was unchanged if the solutions (0.5 mg of protein/ml) were incubated overnight at 4°C with 1% 2-mercaptoethanol, but was somewhat decreased on incubation at 37°C for 20–60 min with insolubilized trypsin or chymotrypsin (10 mg/ml).

Ehrlich ascites cells. Crotin and curcin (crude, I and II) at concentrations up to 100 µg/ml had almost no effect on protein synthesis by Ehrlich ascites cells (results not shown). No inhibition was observed when crotin II (100 µg/ml) was tested on the same cells in the presence of poly-L-ornithine (10 µg/ml), which increases uptake by cells (Ryser & Hancock, 1965).

Table 1. *Toxicity of crotin and curcin*

Four to seven scalar doses of each preparation were administered to groups of four mice for each dose. The acute LD₅₀ was evaluated at 72 h for crotin and at 48 h for curcin, and the delayed LD₅₀ was evaluated at 7 days. Other details are described in the Experimental section.

Preparation	Range tested (mg/mouse)	Acute LD ₅₀ (mg/mouse)	95% confidence limits (mg/mouse)	Delayed LD ₅₀ (mg/mouse)	95% confidence limits (mg/mouse)
Crude crotin	1.36–5.80	3.37	2.22–5.13	1.87	1.14–3.07
Crotin I	0.53–2.99	1.33	0.90–1.95	0.92	0.60–1.40
Crotin II	1.43–4.62	4.38	2.29–8.39	1.68	0.90–3.12
Crude curcin	2.34–13.22	9.11	6.95–11.96	5.83	3.49–9.75
Curcin I	1.42–10.68	6.48	5.12–8.19	2.21	1.19–2.69

Fig. 1. Effect of crotin on protein synthesis by a reticulocyte lysate

The reaction mixture contained, in a final volume of 0.5 ml: 10 mM-Tris/HCl buffer, pH 7.4, 100 mM-ammonium acetate, 2 mM-magnesium acetate, 1 mM-ATP, 0.2 mM-GTP, 15 mM-creatine phosphate, 25 μg of creatine kinase, 0.05 mM amino acids (minus leucine), 1.5 μCi of L-[^{14}C]leucine, the appropriate amount of inhibitor and 0.2 ml of reticulocyte lysate. Incubation was at 28°C for 5 min. Radioactivity was determined on 25 μl samples. (a) Crude crotin; (b) crotin I; (c) crotin II.

Fig. 2. Effect of curcin on protein synthesis by a reticulocyte lysate

Experimental conditions were as described in the legend to Fig. 1. (a) Crude curcin; (b) curcin I; (c) curcin II.

Discussion

The diversity of the lesions brought about in the animals and the different properties of the various protein peaks obtained from the *Croton tiglium* and *Jatropha curcas* seeds indicate (i) that the active proteins obtained from Croton are different from those obtained from Jatropha, and (ii) that the

active component(s) of peak I is different from that of peak II from either seed, since the former is more toxic and brings about different symptoms and lesions *in vivo*, whereas the latter is much more active on protein synthesis *in vitro*. The possibility cannot be excluded that crotin II and curcin II are natural derivatives (or artifacts) respectively of crotin I and curcin I, which on Sephadex behave as larger proteins.

As outlined in the introduction, this investigation was prompted by the similarities noted in the past between the toxic effects of Croton and Jatropha and the effects of Ricinus and Abrus. Our results show that the extracts from all these seeds share the common property of inhibiting protein synthesis to a comparable extent, but also show some differences, the most impressive perhaps being the degree of toxicity. Croton II and curcin II, although probably not pure, have an inhibitory effect on protein synthesis *in vitro* which is comparable with that of pure ricin and abrin in the same system (Olsnes & Pihl, 1972), and yet they are more than 1000 times less toxic than ricin and abrin (Olsnes *et al.*, 1974a). This could be due, at least in part, to an inability of croton and curcin to penetrate cell walls, as indicated by the fact that these proteins do not affect protein synthesis by Ehrlich ascites cells.

The activity of croton and curcin in cell-free systems is not increased by treatment with 2-mercaptoethanol, which greatly enhances the inhibitory effect of ricin and abrin by splitting their molecules into an effector (A) and a carrier (B) moiety (Olsnes *et al.*, 1974a); moreover, unlike ricin, croton and curcin do not agglutinate Ehrlich ascites cells. All this, and the fact that crotons, at least, do not bind to Sepharose suggests that croton and curcin enter cells with difficulty, because they lack a carrier moiety or at least the galactose-binding groups by which ricin binds to cell membranes (Olsnes *et al.*, 1974b).

Note Added in Proof (Received 10 February 1976)

After this paper had been accepted, we became aware that lesions similar to those that we observed had been described in mice (Adam, 1974) and in goats (Adam & Magzoub, 1975) poisoned with seeds of *Jatropha curcas*.

We thank Professor E. Bonetti for interest and encouragement, Dr. N. Montanaro for advice and help with the statistical calculations, Dr. F. Spreafico for a kind gift of Ehrlich ascites tumours. The work was aided by grants from the Consiglio Nazionale delle Ricerche, Rome.

References

Adam, S. E. I. (1974) *Toxicology* 2, 67-76

- Adam, S. E. I. & Magzoub, M. (1975) *Toxicology* 4, 347-354
- Allen, E. H. & Schweet, R. S. (1962) *J. Biol. Chem.* 237, 760-767
- Carrasco, L., Fernandez-Puentes, C. & Vazquez, D. (1975) *Eur. J. Biochem.* 54, 499-503
- Derenzini, M., Bonetti, E., Marinozzi, U. & Stirpe, F. (1976) *Virchows Arch. B* in the press
- Diem, K. & Lentner, C. (eds.) (1972) *Scientific Tables*, 7th edn., pp. 109-123, Ciba-Geigy S.A., Basle
- Finney, D. J. (1964) *Probit Analysis*, 2nd edn., pp. 122-159, University Press, Chicago
- Flexner, S. (1897) *J. Exp. Med.* 2, 197-216
- Hara, K., Ishiguro, M., Funatsu, G. & Funatsu, M. (1974) *Agric. Biol. Chem.* 38, 65-70
- Irwin, S. (1964) in *Animal and Clinical Pharmacologic Techniques* (Nodine, J. H. & Siegler, P. E., eds.), pp. 36-54, Year Book Medical Publishers, Chicago
- Irwin, S. (1968) *Psychopharmacologia* 13, 222-257
- Jacoby, M. (1924) in *Pharmakologie* (Heffter, A., ed.), vol. 2, pp. 1735-1747, Springer, Berlin
- Kraemer, H. (1915) *Scientific and Applied Pharmacognosy*, pp. 475-476, J. Wiley and Sons, New York
- Lin, J.-Y., Liu, K., Chen, C.-C. & Tung, T.-C. (1971) *Cancer Res.* 31, 921-924
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* 193, 265-275
- Montanaro, L., Sperti, S. & Stirpe, F. (1973) *Biochem. J.* 136, 677-683
- Montanaro, L., Sperti, S., Mattioli, A., Testoni, G. & Stirpe, F. (1975) *Biochem. J.* 146, 127-131
- Nicolle, M. & Cesari, E. (1913) *Ann. Inst. Pasteur Paris* 27, 358-365
- Nicolson, G. L. & Blaustein, J. (1972) *Biochim. Biophys. Acta* 266, 543-547
- Olsnes, S. & Pihl, A. (1972) *FEBS Lett.* 20, 327-329
- Olsnes, S. & Pihl, A. (1973) *Biochemistry* 12, 3121-3126
- Olsnes, S., Refsnes, K. & Pihl, A. (1974a) *Nature (London)* 249, 627-631
- Olsnes, S., Saltvedt, E. & Pihl, A. (1974b) *J. Biol. Chem.* 249, 803-810
- Pappenheimer, A. M., Jr. & Gill, D. M. (1973) *Science* 182, 353-358
- Refsnes, K., Olsnes, S. & Pihl, A. (1974) *J. Biol. Chem.* 249, 3557-3562
- Ryser, H. J.-P. & Hancock, R. (1965) *Science* 150, 501-503
- Schwartz, A. E. (1963) *Prog. Chem. Toxicol.* 1, 385-401
- Sperti, S., Montanaro, L., Mattioli, A. & Stirpe, F. (1973) *Biochem. J.* 136, 813-815
- Waller, G. R., Ebner, K. E., Scroggs, R. A., Das Gupta, B. R. & Corcoran, J. B. (1966) *Proc. Soc. Exp. Biol. Med.* 121, 685-691
- Wehmer, C. (1931) *Die Pflanzenstoffe*, vol. 2, pp. 668-700, Fischer, Jena