Bryodin, a ribosome-inactivating protein from the roots of *Bryonia dioica* L. (white bryony)

Fiorenzo STIRPE,* Luigi BARBIERI,* Maria Giulia BATTELLI,* Anna Ida FALASCA,†
Ada ABBONDANZA,* Enzo LORENZONI* and William A. STEVENS†

*Istituto di Patologia generale and †Istituto di Chimica biologica dell'Università di Bologna, I-40126 Bologna, Italy, and

‡Department of Botany, Royal Holloway College, University of London, Huntersdale, Callow Hill, Virginia Water, Surrey GU25 4LN, U.K.

1. Bryodin is a strongly basic ($pI \geq 9.5$) glycoprotein (neutral sugar content 6.3%) with M_r 30000, purified from the roots of *Bryonia dioica* (white bryony). 2. This protein inhibits protein synthesis by a rabbit reticulocyte lysate with an ID_{50} (concentration causing 50% inhibition) of 0.12 nM (3.6 ng/ml) and has much less effect on protein synthesis by whole cells, with ID_{50} values ranging from 46 nM to 2.27 μ M (1.4–67 μ g/ml). 3. Bryodin acts by inactivating ribosomes, with a less-than-equimolar ratio, which suggests a catalytic action. 4. Bryodin decreases the number of local lesions induced by tobacco mosaic virus in the leaves of *Nicotiana glutinosa*. 5. From all its properties, bryodin can be considered to be a ribosome-inactivating protein, similar to those already known [reviews: Barbieri & Stirpe (1982) *Cancer Surveys* 1, 489–520; Stirpe & Barbieri (1986) *FEBS Lett.* 195, 1–8].

INTRODUCTION

Many plants contain ribosome-inactivating proteins (RIPs) type 1 which, like the A-chains of ricin and related toxins (RIPs type 2), damage eukaryotic ribosomes, thus arresting protein synthesis (reviewed by Barbieri & Stirpe, 1982; Stirpe & Barbieri, 1986). The first RIP type 1, namely the pokeweed antiviral protein (PAP), was detected as an anti-viral protein in the leaves of *Phytolacca americana* (pokeweed) (reviewed by Irvin, 1983). Subsequently, it was found that RIPs are present in several parts of many, and possibly all, plants, including seeds, roots, leaves and latices (Gasperi-Campani *et al.*, 1977, 1980, 1985). The highest concentrations were found in plants belonging to the families of Caryophyllaceae, Cucurbitaceae, Euphorbiaceae and Phytolaccaceae.

Apart from their own biological significance, RIPs have attracted considerable attention for their use in the construction of conjugates with antibodies ('immunotoxins'), selectively toxic to the cells bearing the antigens related to the antibodies used (Lord *et al.*, 1985).

In the present experiments we examined materials (mostly seeds) from several members of the Cucurbitaceae family and found that virtually all of them inhibited protein synthesis, and that those examined further contained at least one inhibitory protein. One of these, which we propose to call 'bryodin', was purified from the roots of *Bryonia dioica* L. (white bryony) and was partially characterized. Bryodin is a glycoprotein that in its physicochemical characteristics and biological properties is very similar to the RIPs type 1 already known, and particularly to those purified from other Cucurbitaceae [momordin (Barbieri *et al.*, 1980); luffin (Kishida *et al.*, 1983)].

EXPERIMENTAL

Materials

Plant materials were obtained from the sources indicated in Table 1. L-[14 C]Leucine and L-[14 C]phenylalanine were from Amersham International, Amersham, Bucks., U.K. Materials for chromatography and SPDP were from Pharmacia AB, Uppsala, Sweden. Poly(U) and tRNA were from Sigma Chemical Co., St. Louis, MO, U.S.A. Phenylalanine tRNA was prepared as described by Hultin & Näslund (1978). All other chemicals were as in previous work (Stirpe *et al.*, 1983). Viscumin was kindly given by Dr. P. Ziska, Berlin (D.D.R.). Other RIPs were prepared as described in the respective references: PAP-S (Barbieri *et al.*, 1982), saporin 6 (Stirpe *et al.*, 1983), gelonin (Stirpe *et al.*, 1980), momordin (Barbieri *et al.*, 1980), ricin (Nicolson *et al.*, 1974), abrin (Wei *et al.*, 1974), modeccin (Gasperi-Campani *et al.*, 1978), volkensin (Stirpe *et al.*, 1985). Rabbit antisera against RIPs were raised as described by Falasca *et al.* (1982).

Preparation of extracts and purification of ribosome-inactivating proteins

Seeds were ground with an Ultra-Turrax apparatus with phosphate-buffered saline (0.14 M-NaCl/5 mM-sodium phosphate buffer, pH 7.4) (8 ml/g of seed). Roots and leaves were ground in a blender with 2–3 vol. of the same solution. After overnight stirring at 4 °C, the extracts were strained through cheesecloth or passed through a kitchen centrifuge, and were then centrifuged at 28000 g for 30 min at 2 °C. The supernatants, referred to as 'crude extracts', were dialysed for at least 24 h at 4 °C against 50–100 vol. of 5 mM-sodium phosphate

Abbreviations used: RIP(s), ribosome-inactivating protein(s); PAP, pokeweed antiviral peptide; ID_{50} , concentration giving 50% inhibition; SPDP, N-succinimidyl 3-(2-pyridyldithio)propionate.

Table 1. Inhibitory activity of extracts and purified proteins from Cucurbitaceae on protein synthesis by a rabbit reticulocyte lysate

The purification procedure is described in the text. Reaction mixtures contained, in a final volume of 62.5 μ l: 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-phosphocreatine, 3 μ g of creatine kinase, 0.05 mM amino acids (minus leucine), 89 nCi of L-[14 C]leucine, and 25 μ l of a rabbit reticulocyte lysate prepared as described by Allen & Schweet (1962). Incubation was at 28 °C for 5 min. The reaction was arrested with 1 ml of 0.1 M-KOH, and the radioactivity incorporated into protein was measured as described by Gasperi-Campani *et al.* (1978). One unit of activity is defined as the amount giving 50% inhibition of protein synthesis in 1 ml of reticulocyte lysate. The sources of materials were: (1) Botanical Garden, University of Padua, Italy; (2) Botanical Garden, University of Bologna, Italy; (3) United Chemicals and Allied Products, Calcutta, India; (4) Botanical Garden, University of London, Egham, Surrey, U.K.

Species (source)	Part used	Preparation	Protein (mg/100 g of starting material)	Activity	
				Specific ($10^{-3} \times$ units/ mg)	Total ($10^{-6} \times$ units/ 100 g of starting material)
<i>Benincasa hispida</i> (1)	Seeds	Crude	9120	0.11	1.04
<i>Bryonia dioica</i> (2)	Leaves	Crude	809	2.83	2.29
		CM 0.097 M*	0.5	128	0.064
		CM 0.112 M	1.2	222	0.267
	Roots	Crude	1120	6.99	7.83
		CM 0.033 M	4.9	98.0	0.48
		CM 0.054 M	3.7	99.0	0.37
		CM 0.100 M	4.9	141	0.69
(Large-scale preparation)	Roots	CM 0.100 M	6.9	278	1.92
<i>Cyclonthera explodens</i> (1)	Seeds	Crude	5040	0.77	0.39
<i>Cyclonthera pedata</i> (1)	Seeds	Crude	3770	5.44	20.5
<i>Echinocystis lobata</i> (1)	Seeds	Crude	9040	8.93	80.7
<i>Echinocystis wrightii</i> (1)	Seeds	Crude	5100	9.52	48.6
<i>Lagenaria siceraria</i> (1)	Seeds	Crude	4960	0.53	2.63
<i>Luffa acutangola</i> (3)	Seeds	Crude	1430	4.70	6.72
		CM 0.106 M	7.8	112	0.87
<i>Luffa aegyptiaca</i> (4)	Seeds†	Crude	7960	8.62	68.6
<i>Luffa purgans</i> (1)	Seeds	Crude	7220	3.52	25.4
<i>Sicyos angulatus</i> (4)	Seeds	Crude	4339	6.94	30.1
		CM 0.185 M	72	59.2	4.26

* CM followed by a molarity indicates a peak eluted from a CM-Sepharose column at that molarity of the NaCl gradient.

† Shelled seeds.

buffer, pH 6.5, with at least two changes. Any precipitate that formed during dialysis was removed by centrifugation as above, and the clear extracts were applied to a CM-Sepharose Fast Flow column (20 cm $\times$ 2 cm or 20 cm $\times$ 1.6 cm), previously equilibrated with the same 5 mM buffer. After washing, the column was eluted with a 0–0.3 M-NaCl gradient in the same buffer (total gradient volume 1000 ml for the 2 cm column and 500 ml for the 1.6 cm column).

Bryony root RIP was prepared on a much larger scale as described in the legend to Fig. 1.

Chemical determinations

M_r values were determined by the polyacrylamide-gel-electrophoresis method of Laemmli (1970), with the following markers (all from Bio-Rad Laboratories s.r.l., Segrate, MI, Italy): lysozyme (M_r 14400), soya-bean trypsin inhibitor (21500), carbonic anhydrase (31000), ovalbumin (45000), bovine serum albumin (66200) and

phosphorylase *b* (92500). M_r values were also determined by gel filtration through a column (95 cm $\times$ 2.6 cm) of Sephacryl S-200, equilibrated with 0.3 M-NaCl/5 mM-sodium phosphate buffer, pH 7.2, and eluted at 36 ml/h, at room temperature (20 °C). The column was calibrated with bovine serum albumin (M_r 68000), ovalbumin (43000), chymotrypsinogen (25000) and ribonuclease (13700).

The pI and the amino acid, amino sugar and neutral sugar compositions of the protein were determined as described by Falasca *et al.* (1982).

Proteinase activity, with Azocoll (Calbiochem-Behring, San Diego, CA, U.S.A.) as a substrate, and ribonuclease activity, with yeast RNA (type XI, Sigma) as a substrate, were determined as described by Nelson *et al.* (1981) and by Razzel (1963) respectively.

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin (Sigma) as a standard, or spectrophotometrically (Kalb & Bernlohr, 1977).

Fig. 1. Large-scale chromatography of bryodin

Bryonia roots (usually 2–3 kg) were cut into small pieces, which were shredded and centrifuged in a Braun kitchen shredder-centrifuge to extract their juice. The solid residue was stirred with a volume of the phosphate-buffered saline described in the text equal to the volume of the juice extracted, and the suspension was passed through the same kitchen centrifuge. The combined juice and extract were centrifuged at 10000 *g* for 45 min at 2 °C, and the supernatant was saturated with solid $(\text{NH}_4)_2\text{SO}_4$. The precipitate was collected by centrifugation as described above and was redissolved in 1.5 litres of 5 mM-sodium phosphate buffer, pH 6.66. This solution was clarified by centrifugation as above and was applied to a Sephadex G-25 (Coarse) column (1 m $\times$ 10 cm), previously equilibrated with the 5 mM-sodium phosphate buffer at 20 °C. The column was eluted with the same buffer at a rate of 4 l/h, and the front protein-containing peak was collected and centrifuged and was then applied to a CM-Sephacrose Fast Flow column (15 cm $\times$ 10 cm) at 20 °C. After washing with the sodium phosphate buffer, the column was eluted at the rate of 1.0 l/h with a 0–150 mM-NaCl gradient in the same buffer (total volume 20 l). Fractions (400 ml) were collected, and those containing protein-synthesis-inhibitory activity were pooled, dialysed extensively against water, and freeze-dried.

Preparation of 2-pyridyldithiopropionylated bryodin

Bryodin was dissolved in 0.14 M-NaCl/5 mM-sodium phosphate buffer, pH 7.5, at a concentration of 10 mg/ml. To portions of this solution different amounts of SPDP (1 mg/0.1 ml of *NN*-dimethylformamide) were added. After 50 min at 25 °C the excess of reagent was removed by gel filtration on a Sephadex G-25 (superfine) column (1.6 cm $\times$ 26 cm). The content of 2-pyridyl disulphide groups in the modified bryodin was determined as described by Carlsson *et al.* (1978).

Cell-free protein synthesis

Protein synthesis was measured with a rabbit reticulocyte lysate as described previously (Stirpe *et al.*, 1983).

Poly(U)-directed phenylalanine polymerization was determined with ribosomes purified from a rabbit reticulocyte lysate (Montanaro *et al.*, 1978) and with ribosomes purified from wheat germ or from *Bryonia dioica* leaves as described by Battelli *et al.* (1984). Experimental details are given in the legends to the relevant Tables in the Results section. The ID_{50} (concentration giving 50% inhibition) was calculated by linear-regression analysis.

Effects on cells

The toxicity of bryodin to cells was evaluated (i) from the inhibition of protein synthesis by JAR cells, TG cells, BHK cells, human fibroblasts and mouse peritoneal macrophages and (ii) from the inhibition of DNA synthesis by phytohaemagglutinin-stimulated human peripheral lymphocytes. TG cells (human oviduct carcinoma), JAR cells (human choriocarcinoma), BHK cells (baby hamster kidney) and human fibroblasts were maintained as monolayer cultures in RPMI 1640 medium supplemented with non-essential amino acids, antibiotics and 10% foetal-calf serum. Mouse peritoneal macrophages were collected after intraperitoneal injection of 2 ml of 1% hydrolysed starch solution as described by Stuart *et al.* (1973). Cells were seeded into trays with 2 cm wells, at 0.5×10^5 – 1×10^5 cells/well. The following day the medium was replaced by serum-free RPMI 1640 medium with different amounts of bryodin. After 18 h protein synthesis was measured by incubating the cells for 1–5 h in serum- and leucine-free RPMI 1640 containing 0.1–1 μCi of L-[^{14}C]leucine/ml. The radioactivity incorporated into protein was determined as described by Sandvig & Olsnes (1982).

DNA synthesis by human peripheral lymphocytes was measured as described by Licastro *et al.* (1980).

Anti-viral activity

A crude suspension of tobacco necrosis virus was obtained by grinding frozen samples of highly infected leaves of *Phaseolus vulgaris* var. The Prince in 10 mM-sodium phosphate buffer, pH 7.0 (5 ml/g of leaves). The extract was centrifuged at 5000 *g* for 15 min, and the supernatant was diluted with buffer to give about 50 local lesions per leaf on *P. vulgaris* leaves. Tobacco mosaic virus was purified from infected leaves of *Nicotiana tabacum* var. Xanthii as described by Gooding & Herbert (1967) and was diluted to give about 50 lesions per leaf on leaves of *Nicotiana glutinosa*. The anti-viral activity of bryodin was evaluated from the decrease in the number of local lesions obtained by infecting leaves as described by Stirpe *et al.* (1981) in the presence of bryodin.

Toxicity

The toxicity of the purified protein was evaluated in Swiss female mice weighing 27–32 g, fed *ad lib*. The protein, dissolved in 0.9% NaCl, was injected intraperitoneally at six different dosages ranging from 5.6 to 23.7 mg/kg body wt., with a ratio between doses of 1.33, to groups of six animals for each dose. An acute and an acute delayed LD_{50} were calculated at 48 h and 14 days respectively, by the method of Spearman-Kärber as described by Finney (1964).

Table 2. Physico-chemical properties of bryodin

Experimental conditions are described in the text.

M_r			
By gel filtration 30000			
By gel electrophoresis 27000			
pI $\geq$ 9.5			
Amino acid	Composition* (mol/mol)	Sugar content	
Lys	8.6	Total neutral sugar 6.33%	
His	1.9		
Arg	11.8	Sugar	Composition* (mol/mol)
Asx	22.5		
Thr†	15.1		
Ser†	30.2		
Glx	17.7		
Pro	6.7	Fuc	3.47
Gly	11.4	Glc	1.55
Ala	22.4	Man	6.27
‡-Cys‡	0.24	Xyl	0.93
Val	15.6		
Met§	1.6		
Ile	15.1		
Leu	28.3		
Tyr†	14.2		
Phe	8.3		
Trp	2.0		

* An M_r of 30000 has been considered.

† Values obtained from the hydrolysis at 24, 48 and 72 h were extrapolated to zero time.

‡ Determined as cysteic acid and methionine sulphone.

§ Determined as described by Edelhoch (1967).

RESULTS

Inhibitory activity of extracts and purification of bryodin

All extracts tested had inhibitory activity on cell-free protein synthesis, although to a highly variable extent, ranging over two orders of magnitude (Table 1).

The most active extracts from materials available in sufficient amounts were subjected to the ion-exchange-chromatography procedure used to purify other RIPs (Gasperi-Campani *et al.*, 1985). Up to four protein peaks with protein-synthesis-inhibitory activity were isolated from each extract (Table 1). On SDS/polyacrylamide-gel electrophoresis these peaks showed one major band with mobility corresponding to M_r approx. 30000, sometimes with small amounts of other proteins.

Bryony roots were the most abundant material tested and could be used for the preparation of large quantities of the RIP that they contained. The purification procedure was adapted for larger-scale work as described in the legend to Fig. 1. The major active protein peak obtained by CM-Sephadex chromatography (Fig. 1) was homogeneous, as shown by SDS/polyacrylamide-gel electrophoresis, and the protein so obtained is referred to as 'bryodin'.

Physico-chemical properties of bryodin

The properties of purified bryodin are summarized in Table 2. Like other RIPs (Stirpe & Barbieri, 1986),

bryodin is a glycoprotein with M_r 30000 and strongly basic pI.

The amino acid and neutral sugar compositions are also given. As compared with other RIPs, bryodin has a lower content of lysine and especially of sulphur amino acids, and a higher content of serine and, to a lesser extent, of leucine and tyrosine.

Bryodin did not form any precipitate in an Ouchterlony test with rabbit antisera against other RIPs (PAP, gelonin, momordin, dianthin 30 and dianthin 32).

The protein-synthesis-inhibitory activity of bryodin (see below) was not abolished by overnight incubation at 37 °C in the presence of trypsin or chymotrypsin, as happened with other RIPs (Stirpe *et al.*, 1983), was slightly impaired by treatment with urea or *N*-ethylmaleimide in the presence of urea, and was completely abolished by boiling or by treatment with SDS or with 0.1 M-NaOH (Table 3).

The introduction of 1.25 2-pyridyl disulphide groups per bryodin molecule decreased to one-third the inhibitory activity of the modified bryodin on cell-free protein synthesis (Table 3). Bryodin had no unspecific proteinase or ribonuclease activity, at concentrations up to 100 µg/ml.

Inhibition of protein synthesis

Cell-free systems. Bryodin is a potent inhibitor of protein synthesis by a rabbit reticulocyte lysate (Table 1), with an ID_{50} of 3.6 ng/ml (0.12 nM). The concentration of ribosomes in the assay mixture was 64 nM, as determined from the ribosomes recovered by centrifugation of the lysate and measured as described by Stirpe *et al.* (1981). Assuming a complete recovery of totally active ribosomes, one molecule of bryodin inactivated 265 ribosomes in 5 min, and thus bryodin acted in a less-than-equimolar ratio.

Bryodin also inhibited poly(U)-directed polyphenylalanine synthesis by purified reticulocyte ribosomes, either when it was present during the assay (Fig. 2) or when it was preincubated with ribosomes, which were then washed before assay (Table 4). In both cases bryodin was again inhibitory at concentrations less-than-equimolar with respect to ribosomes.

Bryodin had little effect on wheat-germ ribosomes, and no effect on ribosomes from bryony leaves. These were inactivated by some other RIPs, although at concentrations higher than those effective on wheat-germ ribosomes (Battelli *et al.*, 1984) (Table 5).

Cells. Bryodin affected protein synthesis by various cells, but at much higher concentrations than those inhibitory in cell-free systems (Table 6). It is noteworthy that the inhibitory effect was greater on peritoneal macrophages than on other cells tested. Bryodin was also toxic to human peripheral lymphocytes, as judged from its effect on DNA synthesis (Table 6).

Toxicity

Intraperitoneally injected bryodin killed mice with an LD_{50} of 14.5 mg/kg body wt. (95% confidence limits 11.7–19.2) at 48 h, and of 12.1 mg/kg (9.3–15.7) at 14 days. Histological examination showed signs of necrosis in the liver, in the red pulp of the spleen and in the proximal tubules of the kidneys of dead animals.

Table 3. Effect of various treatments on the protein-synthesis-inhibitory activity of bryodin

Treatments of bryodin were as follows. Expt. 1: the protein (400 $\mu\text{g/ml}$) was incubated in 20 mM-sodium phosphate buffer, pH 7.4 (a), or in water (b), with or without the indicated additions. Expt. 2: the protein (20 $\mu\text{g/ml}$) was incubated in 50 mM-sodium phosphate buffer, pH 7.4, with or without trypsin or chymotrypsin (2 $\mu\text{g/ml}$). Expt. 3: conditions are described in the text. The inhibitory effect on protein synthesis was assayed with the lysate system described in the legend to Table 1, with bryodin at a concentration of 10 ng/ml.

Expt. no.	Additions and treatments	Protein synthesis	
		(d.p.m. incorporated)	(% of controls)
1	Controls	3920	(100)
	(a) Bryodin kept at 37 °C for 30 min with:		
	No additions	895	23
	8 M-Urea	1800	46
	1.7 mM- <i>N</i> -Ethylmaleimide	1320	36
	8 M-Urea and 1.7 mM- <i>N</i> -ethylmaleimide	2800	71
	(b) Bryodin kept at 37 °C overnight with:		
	No additions	1320	33
	0.1% SDS	4160	106
	0.1 M-NaOH	3740	96
	0.1 M-HCl	2200	56
2	Controls	3760	(100)
	Bryodin, boiled	3910	103
	Bryodin kept at 37 °C overnight with:		
	No additions	1130	30
	Trypsin	1320	35
3	Chymotrypsin	1100	20
	Controls	3250	100
	Bryodin	400	12
	Bryodin, SPDP-treated	1200	37

Anti-viral activity

Bryodin mixed with viral suspensions before infection decreased the number of lesions produced by tobacco necrosis virus in the leaves of *Phaseolus vulgaris* (by 77% at 5 μg of bryodin/ml) and by tobacco mosaic virus in the leaves of *Nicotiana glutinosa* (by 72% at 1 μg of bryodin/ml).

DISCUSSION

All crude extracts examined inhibited cell-free protein synthesis, some of them at very low concentrations. Whenever a purification was attempted, one or more proteins could be separated by CM-Sepharose chromatography which were potent inhibitors of protein synthesis, resembling previously identified RIPs. This was confirmed by the properties of bryodin, the protein that could be purified in large quantity from the roots of white bryony. Bryodin has a M_r and a pI similar to those of other RIPs, and like many of these is a glycoprotein. On the other hand, it has a different amino acid composition and appears immunologically distinct from other RIPs, including momordin from *Momordica charantia*, a plant belonging to the same family, the Cucurbitaceae. It should be recalled that bryony seed extracts inhibit cell-free protein synthesis, which suggests that they contain bryodin (Gasperi-Campani *et al.*, 1977).

Bryodin inhibits protein synthesis by damaging

ribosomes in the absence of any cofactor, and acting in a less-than-equimolar ratio, indicating a catalytic (enzymic?) action. As with other RIPs, the effect of bryodin was maximum on reticulocyte ribosomes. Ribosomes from wheat germ and from bryony leaves were scarcely or not at all inhibited by bryodin at concentrations some four orders of magnitude higher than those inactivating reticulocyte ribosomes. Since ribosomes from both wheat germ and bryony leaves were sensitive to other RIPs (Battelli *et al.*, 1984; the present work), our results confirm that RIPs have different effects on plant ribosomes, which indicates a sort of specificity in the mechanism of action of these proteins. This and the different effect of RIPs on ribosomes from protozoa (Villemez *et al.*, 1986) suggests that RIPs, although acting on ribosomes in an apparently similar manner, are actually different from each other in several respects. Thus these proteins may be useful research tools, for instance to pinpoint differences among ribosomes. The effect of bryodin on protein synthesis by cells cultured *in vitro* is similar to that of other RIPs, i.e. much less marked than the effect on cell-free systems. Its higher toxicity to macrophages is also consistent with previous observations with PAP and momordin (Spreafico *et al.*, 1983), and so is its toxicity to lymphocytes (Licastro *et al.*, 1980).

Bryodin seems fairly resistant to several treatments, and in particular retained some 35% of original activity in its 2-pyridyldithiopropionylated form. This makes bryodin suitable for the preparation of immunotoxins or

Fig. 2. Effect of bryodin on phenylalanine polymerization by purified reticulocyte ribosomes

Assays were done with duplicate samples containing, in a final volume of 0.25 ml: 80 mM-Tris/HCl buffer, pH 7.4, 120 mM-magnesium acetate, 2 mM-GTP, 200 μ g of poly(U), 250 μ g (as protein) of 'pH 5 supernatant', 25 pmol of [14 C]phenylalanyl-tRNA and 20 pmol of rabbit reticulocyte ribosomes, calculated as described by Montanaro *et al.* (1978). After incubation at 24 °C for 30 min, 0.25 ml of 10% (w/v) trichloroacetic acid was added, and the acid-insoluble radioactivity was determined as described by Montanaro *et al.* (1978). Control values were 1120 d.p.m.

Table 4. Effect of bryodin on poly(U)-directed phenylalanine polymerization

Rabbit reticulocyte ribosomes (200 pmol) in 1 ml of 80 mM-Tris/HCl buffer, pH 7.4, containing 120 mM-KCl, 7 mM-magnesium acetate and 2 mM-dithiothreitol were preincubated at 24 °C for 30 min, in the presence or in the absence of bryodin. Ribosomes were washed by centrifugation at 105000 g for 3 h through 1.5 ml of 5% sucrose in the same medium. Assays were done as described in the legend to Fig. 2.

Additions		Polyphenylalanine polymerized	
During preincubation (pmol)	During assay (pmol)	(d.p.m. incorporated)	(% of controls)
None	None	2880	(100)
Bryodin (100)	None	350	14
Bryodin (1000)	None	410	12
None	Bryodin (1.0)	370	14
None	Bryodin (0.1)	750	26

of other conjugates, as has been done with other RIPs (reviewed by Stirpe & Barbieri, 1986).

Bryodin has lower intrinsic toxicity to mice than saporin 6, a RIP with which an effective immunotoxin was prepared (Thorpe *et al.*, 1985). Immunotoxins made with saporin 6 were more toxic to animals than was the free protein, and this toxicity appeared to be due to the RIP moiety. Conjugates prepared with an equally active but less toxic RIP may be constructed with bryodin.

Table 5. Effect of bryodin and other ribosome-inactivating proteins on poly(U)-directed polyphenylalanine polymerization by wheat-germ and bryony ribosomes

Ribosomes were prepared as described in the Experimental section. Assays were as described in the legend to Table 4, with 0.73 pmol of wheat-germ ribosomes or with 2.75 pmol of bryony ribosomes. Abrin, ricin, modeccin, viscumin and volkensin, before or after reduction with 1% 2-mercaptoethanol at 37 °C for 1 h, at concentrations up to 1.7 μ M were all inactive on bryony ribosomes.

Ribosomes	Ribosome-inactivating proteins	ID ₅₀ for inhibition of protein synthesis (μ M)
Wheat germ	Bryodin	> 3.3*
	PAP-S	0.018†
Bryony leaves	Bryodin	Inactive‡
	PAP-S	0.012
	Saporin 6	0.008
	Momordin	> 3.3§
	Gelonin	0.82

* 72% of controls at this concentration.
† From Battelli *et al.* (1984).
‡ At 3.3 μ M concentration.
§ 66% of controls at this concentration.

Table 6. Effect of bryodin on protein synthesis by various cells and on DNA synthesis by lymphocytes

Experimental conditions are described in the text.

Cell type	ID ₅₀ (μ M)
Protein synthesis	
TG	0.86
Human fibroblasts	0.90
Mouse peritoneal macrophages	0.15
BHK	2.24
JAR	1.01
DNA synthesis	
Human peripheral lymphocytes	
Unstimulated	0.19
Stimulated with PHA	0.05

We thank Dr. M. Chiricolo for the experiments with lymphocytes, Sig. P. Battistella for excellent technical assistance and Dr. M. Tomaselli, Curator, Sig. G. Bugamelli, Sig. L. Magagnoli and Sig. N. Vicini of the Botanic Garden, University of Bologna, Dr. G. Cassina, Curator of the Botanic Garden, University of Padua, Italy, and the Curator of the University of London Botanic Garden, Egham, Surrey, U.K., for kindly supplying plant materials. The work was supported by the Ministero della Pubblica Istruzione, Rome, by the Pallotti's legacy for Cancer Research, by the Associazione Italiana per la Ricerca sul Cancro and by the Consiglio Nazionale delle Ricerche, Rome, within the progetto finalizzato 'Oncologia' and the Gruppo Nazionale di Coordinamento 'Struttura e Funzione delle Macromolecole biologiche' (Grant no. 85.0594).

REFERENCES

- Allen, E. H. & Schweet, R. S. (1962) *J. Biol. Chem.* **237**, 760–767
- Barbieri, L. & Stirpe, F. (1982) *Cancer Surveys* **1**, 489–520
- Barbieri, L., Zamboni, M., Montanaro, L., Sperti, S. & Stirpe, F. (1980) *Biochem. J.* **186**, 443–452
- Barbieri, L., Aron, G. M., Irvin, J. D. & Stirpe, F. (1982) *Biochem. J.* **203**, 55–59
- Battelli, M. G., Lorenzoni, E., Stirpe, F., Cella, R. & Parisi, B. (1984) *J. Exp. Bot.* **35**, 882–889
- Carlsson, J., Drevin, D. & Axen, R. (1978) *Biochem. J.* **173**, 727–737
- Edelhoc, H. (1967) *Biochemistry* **6**, 1948–1954
- Falasca, A., Gasperi-Campani, A., Abbondanza, A., Barbieri, L. & Stirpe, F. (1982) *Biochem. J.* **207**, 505–509
- Finney, D. J. (1964) *Statistical Methods in Biological Assay*, pp. 524–530, Griffin, London
- Gasperi-Campani, A., Barbieri, L., Lorenzoni, E. & Stirpe, F. (1977) *FEBS Lett.* **76**, 173–176
- Gasperi-Campani, A., Barbieri, L., Lorenzoni, E., Montanaro, L., Sperti, S., Bonetti, E. & Stirpe, F. (1978) *Biochem. J.* **174**, 491–496
- Gasperi-Campani, A., Barbieri, L., Morelli, P. & Stirpe, F. (1980) *Biochem. J.* **186**, 439–441
- Gasperi-Campani, A., Barbieri, L., Battelli, M. G. & Stirpe, F. (1985) *J. Nat. Prod.* **48**, 446–454
- Gooding, G. V. & Herbert, T. T. (1967) *Phytopathology* **57**, 1285
- Hultin, T. & Näslund, P. H. (1978) *Eur. J. Biochem.* **88**, 143–148
- Irvin, J. D. (1983) *Pharmacol. Ther.* **21**, 371–387
- Kalb, V. F., Jr. & Bernlohr, R. W. (1977) *Anal. Biochem.* **82**, 362–371
- Kishida, K., Masuho, Y. & Hara, T. (1983) *FEBS Lett.* **153**, 209–212
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Licastro, F., Franceschi, C., Barbieri, L. & Stirpe, F. (1980) *Virchows Arch. B* **33**, 257–265
- Lord, J. M., Roberts, L. M., Thorpe, P. E. & Vitetta, E. S. (1985) *Trends Biotechnol.* **3**, 175–179
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Montanaro, L., Sperti, S., Zamboni, M., Denaro, M., Testoni, G., Gasperi-Campani, A. & Stirpe, F. (1978) *Biochem. J.* **176**, 371–376
- Nelson, J. A., Levy, J. A. & Leong, J. C. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 1670–1674
- Nicolson, G. L., Blaustein, J. & Etzler, M. E. (1974) *Biochemistry* **13**, 196–204
- Razzel, W. A. (1963) *Methods Enzymol.* **6**, 236–258
- Sandvig, K. & Olsnes, S. (1982) *J. Biol. Chem.* **257**, 7495–7503
- Spreafico, F., Malfiore, C., Moras, M. L., Marmonti, L., Filippeschi, S., Barbieri, L., Perocco, P. & Stirpe, F. (1983) *Int. J. Immunopharmacol.* **5**, 335–344
- Stirpe, F. & Barbieri, L. (1986) *FEBS Lett.* **195**, 1–8
- Stirpe, F., Olsnes, S. & Pihl, A. (1980) *J. Biol. Chem.* **255**, 6947–6953
- Stirpe, F., Williams, D. G., Onyon, L. J., Legg, R. F. & Stevens, W. A. (1981) *Biochem. J.* **195**, 399–405
- Stirpe, F., Gasperi-Campani, A., Barbieri, L., Falasca, A., Abbondanza, A. & Stevens, W. A. (1983) *Biochem. J.* **216**, 617–625
- Stirpe, F., Barbieri, L., Abbondanza, A., Falasca, A. I., Brown, A. N. F., Sandvig, K., Olsnes, S. & Pihl, A. (1985) *J. Biol. Chem.* **260**, 14589–14595
- Stuart, A. E., Habeshaw, J. A. & Davidson, A. E. (1973) in *Handbook of Experimental Immunology* (Weir, D. M., ed.), pp. 24.1–24.6, Blackwell Scientific Publications, Oxford
- Thorpe, P. E., Brown, A. N. F., Bremner, J. A. G., Jr., Foxwell, B. M. J. & Stirpe, F. (1985) *J. Natl. Cancer Inst.* **75**, 151–159
- Villemez, C. L., Russell, M. A., Barbieri, L., Stirpe, F., Irvin, J. D. & Robertus, J. D. (1986) *UCLA Symp. Mol. Cell. Biol. New Ser.* **45**, in the press
- Wei, C.-H., Hartman, F. C., Pfuderer, P. & Yang, W.-K. (1974) *J. Biol. Chem.* **249**, 3061–3067

Received 15 April 1986/29 July 1986; accepted 21 August 1986