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# Modeccin, the Toxin of *Adenia digitata*

## PURIFICATION, TOXICITY AND INHIBITION OF PROTEIN SYNTHESIS *IN VITRO*

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1. Modeccin, the toxin of *Adenia digitata* (*Modecca digitata*), was purified from the roots of this plant by affinity chromatography on Sepharose 4B. 2. This toxin is a protein with mol.wt. 57000, which on treatment with 2-mercaptoethanol can be dissociated into two subunits of mol.wts. 25000 and 32000. 3. Modeccin inhibits protein synthesis *in vitro* in a lysate of rabbit reticulocytes and in Ehrlich ascites cells; the effect on cells is decreased in the presence of lactose. 4. Dissociation of modeccin into subunits decreases the toxicity to animals and the inhibition of protein synthesis in cells, but enhances the inhibition of protein synthesis in the lysate system.

The toxicity to animals and to man of the roots of *Adenia digitata* Burtt-Davy (*Modecca digitata* Harv.), a member of the Passifloraceae growing in southern Africa (Watt & Breyer-Brandwijk, 1962; Buchan & Buchan, 1971), is caused by both a cyanogenic glycoside and particularly a very potent toxin (Green & Andrews, 1923, 1924). The latter investigators partially purified this toxin, called by them modeccin, established that it was a protein, and described its toxic effects on various animals. More recently, modeccin was purified in this laboratory, and it was shown to be a powerful inhibitor of protein synthesis in both Ehrlich ascites-tumour cells and a lysate of rabbit reticulocytes (Stirpe *et al.*, 1978).

We have ascertained that modeccin is a protein of mol.wt. 57000, which on treatment with 2-mercaptoethanol can be dissociated into two unequal subunits. This results in a decreased toxicity to animals, in a decreased inhibition of protein synthesis in whole cells, and in an increased inhibition of protein synthesis in the cell-free system. The effect of modeccin on protein synthesis in cells is decreased also in the presence of lactose. These results indicate that modeccin is a toxin whose properties *in vitro* are very similar to those of ricin and abrin.

## Experimental

### *Materials*

Roots of *Adenia digitata* were obtained from the Department of Botany of the University of Pretoria and from the Department of Experimental and Clinical Pharmacology of the University of the Witwatersrand, Johannesburg, Republic of South Africa, and were stored at 0°C or at −25°C until use. Proteins used as standards for molecular-weight determinations were from Boehringer Mannheim G.m.b.H., Mannheim, West Germany. Bovine serum albumin and RNA (from yeast, type XI) were from Sigma Chemical Co., St. Louis, MO, U.S.A. Acrylamide was from Eastman Kodak Co., Rochester, NY, U.S.A. Cystamine was from Fluka A.G., Buchs, Switzerland. L-[$^{14}$C]Leucine (specific radioactivity 354mCi/mmol), [5,6-$^{3}$H]uridine (specific radioactivity 58Ci/mmol) and [*methyl*-$^{3}$H]thymidine (specific radioactivity 20Ci/mmol) were from The Radiochemical Centre, Amersham, Bucks., U.K. All other chemicals were of analytical grade.

Ehrlich ascites-tumour cells were grown in Swiss mice, and were collected 6–8 days after transplantation. Rabbit reticulocyte lysates were prepared as described by Allen & Schweet (1962).

### *Toxicity experiments*

The toxicity of modeccin was evaluated in male Swiss mice weighing 20–25g and in male Wistar rats weighing 100–120g. Animals were supplied with food and water *ad libitum*. The toxin, dissolved in 0.9% NaCl, was injected intraperitoneally at seven scalar doses, ranging from 0.1 to 4.0$\mu$g/100g body wt. to mice, and from 0.025 to 1.0$\mu$g/100g body wt. to rats. Groups of six animals for each dose were used. $LD_{50}$ was evaluated by the method of Spearman-Kärber as described by Finney (1964).

### *Determination of molecular weight*

The method of Weber & Osborn (1969) for sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was used. Gels contained 7% acrylamide and 0.1%

sodium dodecyl sulphate. Modeccin and RNA polymerase used as a marker were split into their subunits by incubation at 37°C for 2h in 0.01 M-sodium phosphate buffer, pH 7.0, containing 0.1% sodium dodecyl sulphate and 1% 2-mercaptoethanol. Runs were at 5mA per tube for 3.5h at room temperature (25°C). Proteins were stained with Coomassie Blue.

*Protein, RNA and DNA synthesis*

Protein synthesis was determined from the incorporation of [$^{14}$C]leucine as described previously (Stirpe *et al.*, 1978) with a lysate of rabbit reticulocytes or with Ehrlich ascites cells. Reaction mixtures are described in the legends to the appropriate Figures. At the appropriate times 25$\mu$l samples were transferred to 1ml of 0.1 M-KOH. After 30min at room temperature 1ml of 20% (w/v) trichloroacetic acid was added, and the precipitated material was collected on glass-fibre discs (Whatman GF/C), which were washed several times with 5% trichloroacetic acid. Discs were transferred to counting vials with 5ml of methoxyethanol and 10ml of scintillation fluid [0.05% 1,4-bis-(5-phenyloxazol-2-yl)benzene and 0.4% 2,5-diphenyloxazole in toluene].

RNA and DNA syntheses were determined with Ehrlich ascites cells in the same medium as used for protein synthesis, except that [$^{3}$H]uridine and [$^{3}$H]-thymidine were added instead of [$^{14}$C]leucine, and samples were put in 10% trichloroacetic acid.

*Other determinations*

Radioactivity was determined in a Packard Tri-Carb liquid-scintillation spectrometer with an external standard. Counting efficiency was approx. 70% for $^{14}$C and 33% for $^{3}$H.

Protein was measured by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard, or by the method of Warburg & Christian (1941).

Ribonuclease activity was measured as described by Razzel (1963) with yeast RNA as substrate, and proteinase activity by the phenol colour method of Greenberg (1955) with bovine serum albumin as substrate.

Agglutination tests were performed in microtitre plates with serial dilutions (1:1) of the toxin in 50$\mu$l of 0.15 M-NaCl containing 5 mM-sodium phosphate buffer, pH 7.2, and 50$\mu$l of 0.5% rabbit erythrocyte suspension.

## Results

*Purification of modeccin*

Modeccin was purified as described by Stirpe *et al.* (1978) with some modifications. All operations were performed at 4°C. Portions of roots of *Adenia digitata*, peeled of skin, were minced with scissors and were homogenized with a blender or with an Ultra-Turrax apparatus in 0.2 M-NaCl containing 0.005 M-sodium phosphate buffer, pH 7.2, and 0.1 M-cystamine. Cystamine was added to prevent release of the HCN liberated by the hydrolysis of the cyanogenic glycoside present in the roots. The suspension was left overnight with magnetic stirring, and was then filtered through filter paper, with suction, on a Büchner funnel (filtration gives a higher yield of extract than centrifugation). The filtrate was clarified by centrifugation at 11000*g* for 20min. The clear supernatant was adjusted to 65% saturation with solid $(NH_4)_2SO_4$, added slowly with constant stirring. No precipitate was formed below 40% saturation, nor was additional material precipitated if the 65%-satd.-$(NH_4)_2SO_4$ supernatant was adjusted to 100% saturation with additional $(NH_4)_2SO_4$. The precipitate was collected by centrifugation, redissolved in the minimum volume of phosphate-buffered saline (without cystamine) and dialysed against the same solution for at least 24h. This preparation will be referred to as crude modeccin.

The toxin was purified further by affinity chromatography on Sepharose 4B. Crude modeccin was loaded on a column of Sepharose 4B, previously equilibrated with phosphate-buffered saline. The column was washed with the same solution until the $A_{280}$ of the effluent was below 0.1, and then was eluted with 0.2 M-galactose in phosphate-buffered saline. A broad irregular peak (peak I) was eluted in the washing, and a sharp symmetrical peak (peak II, modeccin, see below) was eluted with galactose (Fig. 1). The fractions of each peak were pooled and were dialysed against phosphate-buffered saline. The yield of modeccin was 20–180mg/100g of root.

Modeccin could be freeze-dried or solutions could be stored at −25°C and could be thawed and frozen again several times without loss of activity.

*Properties of modeccin*

*Toxicity.* The material from both peaks I and II were highly toxic when injected intraperitoneally to rats and mice. Poisoned animals died between 8h and 8 days after injection, depending on the dose given. For this reason an acute and a delayed $LD_{50}$ were calculated, at 48h and at 10 days after poisoning respectively (Table 1). The highest toxicity was observed with the material of peak II, subsequently termed modeccin. Modeccin was more toxic to rats than to mice, on a body-weight basis, and brought about pathological changes only in part similar to those observed in animals poisoned with ricin (Flexner, 1897; Waller *et al.*, 1966; Derenzini *et al.*, 1976). Lesions were not the same in rats and mice, the main difference noticed at post-mortem examination being an abundant ascites and hydrothorax observed in rats only.

*Purity, molecular weight and subunits.* Modeccin gave a single band on polyacrylamide-gel electrophoresis, performed either with (Fig. 2*a*) or without sodium dodecyl sulphate (results not shown). When the toxin was incubated at 37°C for 2h in the presence of 1% 2-mercaptoethanol, with or without sodium dodecyl sulphate, the single electrophoretic band was replaced by two faster-migrating bands (Fig. 2*b*), indicating that the molecule was dissociated into two subunits.

The molecular weight of modeccin, as estimated by polyacrylamide-gel electrophoresis, was 57000±460 (mean ± S.E.M. of three determinations). The subunits had mol.wts. of 25000±1340 and of 32000± 940 respectively (Fig. 3). Attempts to isolate the separated subunits were unsuccessful.

*Inhibition of protein synthesis*

Modeccin is a potent inhibitor of protein synthesis in cells and in a cell-free system (Stirpe *et al.*, 1978), and this was confirmed consistently with several preparations of the toxin. The effect on Ehrlich cells was significantly decreased in the presence of 0.1M-lactose (Fig. 4).

Dissociation into subunits changed in opposite ways the effect of modeccin on protein synthesis in cells and in a cell-free system. The inhibitory effect on Ehrlich ascites cells was decreased approx. 100-fold, compared with the undissociated toxin (Fig. 5). On the contrary, the inhibitory effect on the reticulocyte lysate system was increased approx. 30-fold, the $ID_{50}$ (concentration giving 50% inhibition) changing from 3 to 0.1 μg/ml on dissociation of modeccin (Fig. 6).

Dissociation of the molecule also decreased the toxicity of modeccin to animals: an $LD_{50}$ was not determined, but it was observed that rats survived after a dose of 5μg of dissociated modeccin/100g body wt., given intraperitoneally.

The material of peak I, although less toxic than modeccin to animals (Table 1), had a stronger inhibitory effect on the lysate system ($ID_{50}$ 0.57μg/ml), whereas 100μg/ml was required to inhibit protein synthesis in Ehrlich ascites cells. This material was not purified or analysed further, but it was excluded by Sephadex G-100 or G-200 columns.

*Other properties*

Modeccin at a concentration of 10μg/ml did not affect RNA or DNA synthesis in Ehrlich cells for 1h, i.e. 30min after protein synthesis was arrested (results not shown). The toxin had no ribonuclease or proteinase activity at concentrations of 20 and 200μg/ml respectively.

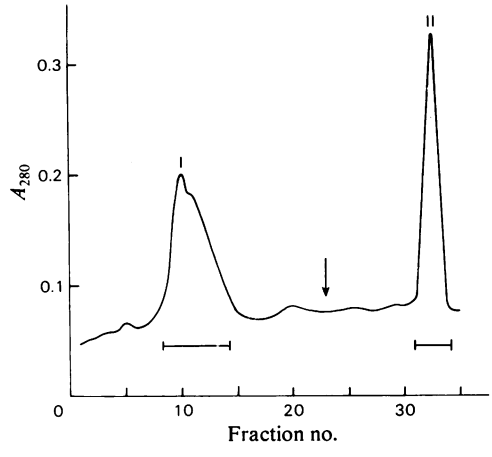


Fig. 1. *Purification of modeccin by chromatography*
Crude modeccin (30mg), dissolved in phosphate-buffered saline, was applied to a column (28cm×1.5cm) of Sepharose 4B, previously equilibrated with the same solution. After washing, the column was eluted with 0.2M-galactose (arrow) in phosphate-buffered saline. Fractions (6ml) were collected and were pooled as indicated by the bars.

Table 1. *Toxicity of modeccin*
Experimental conditions are described in the Experimental section: 95% confidence limits are given in parentheses. Peak numbers refer to Fig. 1.

| | $LD_{50}$ (μg/100g body wt.) | | | |
|---|---|---|---|---|
| | Mice | | Rats | |
| | 48h | 10 days | 48h | 10 days |
| Peak I | Not determined | Not determined | 6.30 (5.02–7.90) | 3.53 (2.36–5.29) |
| Peak II (modeccin) | 0.53 (0.36–0.77) | 0.23 (0.15–0.77) | 0.13 (0.08–0.20) | 0.09 (0.05–0.15) |

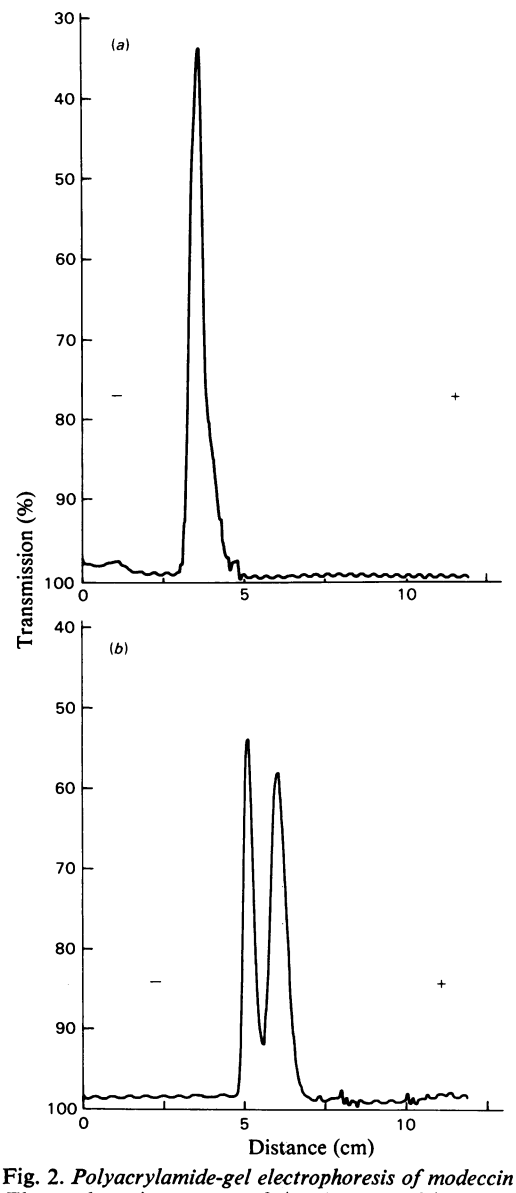


Fig. 2. *Polyacrylamide-gel electrophoresis of modeccin*
Electrophoresis was performed on 10% polyacrylamide gel in the presence of sodium dodecyl sulphate, as described by Weber & Osborn (1969). Runs were at 8mA per tube, at room temperature (25°C), from cathode to anode. Gels were scanned in a Kipp and Zonen densitometer DD2, with a 580–650nm filter. Modeccin was dissociated into subunits by incubation at 37°C for 2h in phosphate-buffered saline containing 1% 2-mercaptoethanol. (*a*) Modeccin; (*b*) dissociated modeccin.

Modeccin (31 $\mu$g/ml) agglutinated rabbit erythrocytes. No agglutination was observed at 15 $\mu$g/ml.

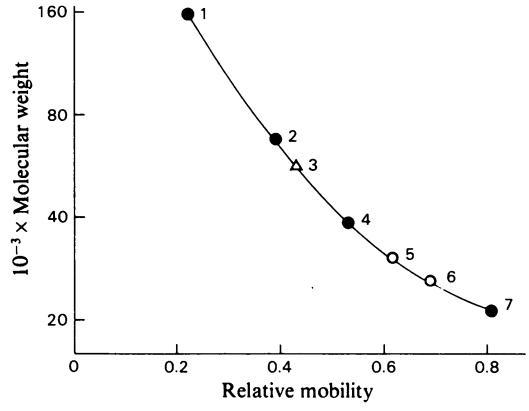


Fig. 3. *Molecular weight of modeccin and of its subunits*
Experimental conditions are described in the Experimental section. 1, $\beta^{-}+\beta^{-\prime}$ subunits of RNA polymerase; 2, bovine serum albumin; 3, modeccin; 4, $\alpha$ subunit of RNA polymerase; 5 and 6, dissociated modeccin; 7, trypsin inhibitor.

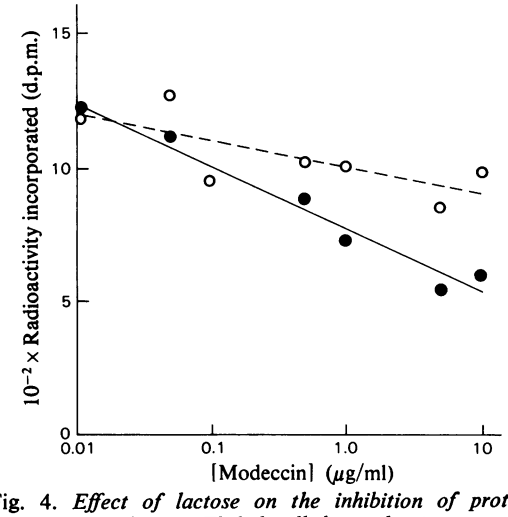


Fig. 4. *Effect of lactose on the inhibition of protein synthesis in Ehrlich cells by modeccin*
Ehrlich ascites cells ($3.3\times10^6$) were incubated at 37°C in 1 ml of medium E 2a (Puck *et al.*, 1957) containing 5% calf serum and 1 $\mu$Ci of L-[$^{14}$C]leucine, without (●) or with (○) 0.1 M-lactose and the appropriate concentration of modeccin. The rate of protein synthesis was estimated from the radioactivity incorporated between 30 and 90 min of incubation. Other details are described in the Experimental section.

## Discussion

Modeccin was purified to homogeneity from the roots of *Adenia digitata*, and is one of the most potent toxins of plant origin. This toxin is a protein

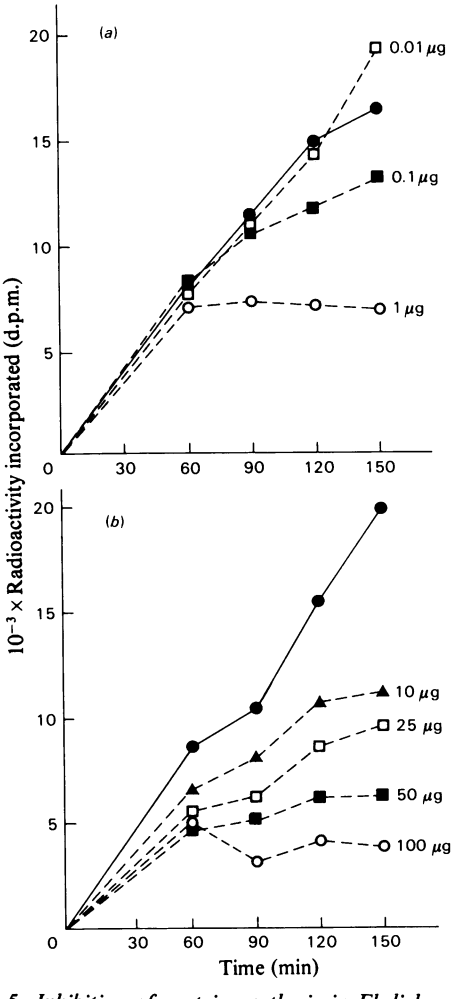


Fig. 5. *Inhibition of protein synthesis in Ehrlich cells by undissociated and dissociated modeccin*
Experimental conditions were as described in the legend to Fig. 4 in the absence (——) or in the presence (- - - -) of undissociated (*a*) or of dissociated (*b*) modeccin at the concentrations shown.

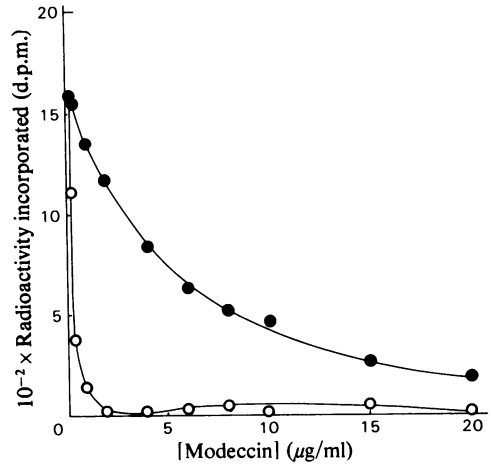


Fig. 6. *Inhibition of protein synthesis in a rabbit reticulocyte lysate by undissociated and dissociated modeccin*
The reaction mixture contained, in a final volume of 0.25 ml: 10 mM-Tris/HCl buffer, pH 7.4, 100 mM-ammonium acetate, 2 mM-magnesium acetate, 1 mM-ATP, 0.2 mM-GTP, 15 mM-phosphocreatine, 12 μg of creatine kinase, 0.05 mM-amino acids (minus leucine), 0.75 μCi of L-[$^{14}$C]leucine, the appropriate amount of undissociated (●) or dissociated (○) modeccin, and 0.1 ml of lysate. Incubation was at 27°C for 5 min. Other details are described in the Experimental section.

with mol.wt. 57000 and consists of two subunits, of mol.wts. 25000 and 32000, joined together by one (or more) disulphide bond(s), as indicated by dissociation with 2-mercaptoethanol.

Green & Andrews (1924), on the basis of the pathology of animals poisoned with semi-purified modeccin, formulated the hypothesis that this toxin could be similar to ricin. Their observations were only in part confirmed by our experiments with pure modeccin, but their remarkable hypothesis was corroborated by the numerous analogies between modeccin on the one hand and ricin and the related toxin abrin on the other (for review of ricin and abrin see Olsnes & Pihl, 1977). These three toxins have a comparable potency *in vivo*, have a similar molecular weight, have some haemagglutinating activity, and inhibit synthesis in cells and in cell-free systems. They can be eluted from Sepharose with galactose, and their effects on cells are decreased in the presence of lactose, which indicates that they bind to galactopyranosyl residues on the cell surface.

Modeccin, like ricin and abrin, is composed of two unequal subunits. The subunits of ricin and abrin have distinct biological functions: the smaller one, called A, damages irreversibly the 60S ribosomal subunit, thus being responsible for the inhibition of protein synthesis, but cannot penetrate inside cells. The subunit B binds to the cell surface, and allows entrance of the A subunit. Dissociation of ricin and abrin into subunits decreases their toxicity to animals or to cells, but enhances their inhibitory effect on cell-free systems. Dissociation of modeccin has similar consequences, and thus it is likely that the subunits of this toxin have properties similar to those of the subunits of ricin and abrin. The fact that modeccin is much less effective than its dissociated subunits in the cell-free system suggests that dissociation facilitates the effect of the subunit active on the protein-synthesizing system, or even that dissociation

may be necessary for this effect to occur. Furthermore, inhibition of protein synthesis in whole cells is obtained with a lower concentration of modeccin as compared with the lysate system, and this suggests that the toxin may be dissociated during or after penetration inside cells.

These results indicate that modeccin is a toxin similar to ricin and abrin, although it brings about different pathological changes in the animals. It is surprising that toxins so similar are present in plants taxonomically far from each other such as *Ricinus communis* (Euphorbiaceae), *Abrus precatorius* (Leguminosae) and *Adenia digitata* (Passifloraceae).

**Note added in Proof (Received 14 March 1978)**

After this paper had been submitted, Refsnes *et al.* (1977) reported the purification of modeccin by a different chromatographic procedure, and described the inhibitory effect of this toxin on protein synthesis by HeLa cells and by a lysate of rabbit reticulocytes.

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