Effect of Modeccin on the Steps of Peptide-Chain Elongation

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Modeccin inhibits polypeptide-chain elongation catalysed by *Artemia salina* (brine shrimp) ribosomes by inactivating the 60S ribosomal subunit. Among the individual steps of elongation, peptide-bond formation, catalysed by 60S peptidyltransferase, is unaffected by the toxin, whereas the binding of EF 2 (elongation factor 2) to ribosomes is strongly inhibited. Modeccin does not affect the poly(U)-dependent non-enzymic binding of either deacylated tRNA^{Phe} or phenylalanyl-tRNA to ribosomes. The inhibitory effect of modeccin on the EF 1 (elongation factor 1)-dependent binding of phenylalanyl-tRNA is discussed, since it is decreased by tRNA^{Phe}, which stimulates the binding reaction. The analysis of the distribution of ribosome-bound radioactivity during protein synthesis shows that modeccin consistently inhibits the radioactivity bound as long-chain peptides, but, depending on the experimental conditions, can leave unchanged or even greatly stimulates the radioactivity bound as phenylalanyl-tRNA and/or short-chain peptides. It is concluded that, during the complete elongation cycle, modeccin does not affect the binding of the first aminoacyl-tRNA to ribosomes, but inhibits some step in the subsequent repetitive activity of either EF 1 or EF 2. The results obtained indicate that the mechanism of action of modeccin is very similar to that of ricin and related plant toxins such as abrin and crotin.

Modeccin is a very potent toxic protein present in the roots of *Adenia digitata*, a Passifloracea growing in southern Africa. Refsnes *et al.* (1977) and Stirpe *et al.* (1978) have shown that the toxin inhibits protein synthesis both in HeLa and Ehrlich ascites cells and in cell-free systems from rabbit reticulo-cytes.

In the present paper the effect of modeccin on the individual steps of polypeptide-chain elongation has been investigated with the use of reconstituted systems of *Artemia salina* (brine shrimp) ribosomes and elongation factors. Modeccin inactivates the 60S ribosomal subunit and inhibits the binding of EF 2 to ribosomes, without affecting the peptidyl-transferase reaction. Thus the effect of modeccin resembles that of ricin and other related plant toxins such as abrin and crotin (Olsnes & Pihl, 1977; Sperti *et al.*, 1976).

Experimental

Materials

Modeccin was prepared as described by Gasperi-Campani *et al.* (1978). Ricin was prepared as described by Nicolson & Blaustein (1972) and Nicolson

Abbreviations used: p[CH₂]ppG, guanosine [β -methylene]triphosphate; EF 1, elongation factor 1; EF 2, elongation factor 2; P site, peptidyl-tRNA site.

et al. (1974) and abrin (abrin C) as described by Wei *et al.* (1974). L-[¹⁴C]Phenylalanyl-tRNA (mixed charged tRNA containing 0.260 μ Ci of L-[¹⁴C]-phenylalanine/mg of tRNA; 414 mCi/mmol of L-phenylalanine; percentage of L-phenylalanine acceptor tRNA bound with L-phenylalanine 36.9%) was purchased from New England Nuclear Corp., Boston, MA, U.S.A. Deacylated tRNA^{Phe} and aminoacyl-tRNA synthetase (55 units/mg of protein; where 1 unit catalyses the activation of 1 nmol of arginine in 10 min at 37°C) were products of Miles Laboratories, Kankakee, IL, U.S.A. [³H]p[CH₂]ppG (5.1 Ci/mmol), [adenosine-¹⁴C]NAD⁺ (260 mCi/mmol) and L-[³H]phenylalanine (1 Ci/mmol) were from The Radiochemical Centre, Amersham, Bucks., U.K. N-Acetyl[³H]phenylalanyl-tRNA was prepared as described by Haenni & Chapeville (1966) from L-[³H]phenylalanyl-tRNA obtained by allowing tRNA^{Phe} to react with L-[³H]phenylalanine in the presence of purified aminoacyl-tRNA synthetase as described by Zasloff & Ochoa (1971).

KCl-washed *A. salina* 80S ribosomes from undeveloped cysts were prepared as described by Sierra *et al.* (1974). *A. salina* ribosomal subunits were prepared as described by Zasloff & Ochoa (1971). In some experiments, the non-washed 80S ribosomes from which subunits were prepared were used. The concentration of ribosomes was calculated from the *A*₂₆₀ with the following assumptions: *A*_{1cm}^{1%} = 125;

1 mg of ribosomes = 250 pmol; 1 mg of 60S subunits = 370 pmol; 1 mg of 40S subunits = 714 pmol. An enzymic fraction containing elongation factors ('s-105 supernatant') was obtained by precipitating the *A. salina* postribosomal supernatant in 75% satd. $(\text{NH}_4)_2\text{SO}_4$ (Sierra *et al.*, 1974). EF 1 and EF 2 were obtained from rat liver 'pH5 supernatant' and resolved from each other as described by Montanaro *et al.* (1973). The concentration of EF 2 was determined by [^{14}C]ADP-ribosylation as described by Bermek (1972). Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Methods

Unless otherwise stated the saline composition of the incubation mixtures was: 80 mM-Tris/HCl buffer, pH 7.4, 120 mM-KCl, 7 mM-magnesium acetate and 2 mM-dithiothreitol (medium A) for poly(U)-directed [^{14}C]phenylalanine polymerization; 80 mM-Tris/HCl buffer, pH 7.4, 80 mM-KCl, 10 mM-magnesium acetate and 2 mM-dithiothreitol (medium B) for the binding reactions; 90 mM-Tris/HCl buffer, pH 7.4, 150 mM-KCl, 7 mM-magnesium acetate and 1.5 mM-dithiothreitol (medium C) for the puromycin reaction.

Poly(U)-directed phenylalanine polymerization. The reaction was carried out at 24°C in 0.25 ml of medium A containing 2 mM-GTP, 25 pmol of [^{14}C]phenylalanyl-tRNA, 200 µg of poly(U), 250 µg of 's-105 supernatant' and 80S ribosomes, or, alternatively, 40S plus 60S ribosomal subunits in the amounts indicated in the legends to the Figures and Tables. After 30 min, the reaction was stopped and the hot acid-insoluble radioactivity was measured as described below.

EF 2-mediated binding of [^3H]p[CH₂]ppG. The reaction was carried out for 20 min at 24°C in 60 µl of medium B containing 10 µM-[^3H]p[CH₂]ppG (diluted with unlabelled carrier to a specific radioactivity of 200 mCi/mmol), 27 pmol of KCl-washed 80S ribosomes and 31 pmol of EF 2 (assayed by [^{14}C]ADP-ribosylation). The EF 2-mediated binding of the nucleotide to ribosomes was calculated by subtracting the amount bound to ribosomes alone and to EF 2 alone from the radioactivity bound when the two components were present together.

Puromycin reactivity of non-enzymically bound *N*-acetyl[^3H]phenylalanyl-tRNA. The non-enzymic binding of *N*-acetyl[^3H]phenylalanyl-tRNA to 40S subunits was carried out as described in the legend to Table 3. The puromycin reactivity of the bound *N*-acetyl[^3H]phenylalanyl-tRNA on addition of 60S subunits and puromycin was determined by measuring the radioactivity extracted in ethyl acetate at pH 5.5 (Zasloff & Ochoa, 1971).

Non-enzymic binding of [^{14}C]phenylalanyl-tRNA. The reaction was carried out for 20 min at 24°C in

100 µl of medium B supplemented with magnesium acetate to a final concentration of 20 mM and containing 20 pmol of 80S ribosomes, 200 µg of poly(U) and 25 pmol of [^{14}C]phenylalanyl-tRNA. Total ribosome-bound radioactivity was measured.

Enzymic binding of [^{14}C]phenylalanyl-tRNA. The reaction was carried out in the presence of EF 1 as described in detail in the legend to Fig. 3.

Radioactivity measurements. Total bound radioactivity. At the end of the incubations, samples were diluted with 5 ml of ice-cold 20 mM-Tris/HCl buffer, pH 7.4, containing 20 mM-KCl and 10 mM-magnesium acetate, and filtered immediately through Millipore filters (HA; average pore size 0.45 µm) kept soaked in the same buffer under reduced pressure for 2 h before use; the filters were washed with 3 × 5 ml of cold buffer.

Hot acid-insoluble radioactivity. At the end of the incubations, the samples were supplemented with an equal volume of 10% (w/v) trichloroacetic acid and were kept for 15 min at 90°C; they were then filtered through glass-fibre filters (Whatman GF/C) and the filters were washed with 5 × 5 ml of 5% (w/v) trichloroacetic acid.

Radioactivity was measured in a Packard Tri-Carb liquid-scintillation spectrometer after adding to the samples 2.5 ml of methoxyethanol and 10 ml of scintillation fluid [0.05% 1,4-bis-(5-phenyloxazol-2-yl)benzene and 0.4% 2,5-diphenyloxazole in toluene]. When a single radioactive isotope was present, the efficiency of counting was 83% for ^{14}C and 40% for ^3H . When ^{14}C had to be measured in the presence of ^3H , the instrument was set for dual-label counting; the spill-over of ^3H into the ^{14}C channel was negligible and the counting efficiency for ^{14}C was 50%. All counts were corrected to 100% efficiency.

Results

Poly(U)-directed phenylalanine polymerization was severely inhibited by modeccin (Fig. 1). Inhibition increased when modeccin was preincubated for 1 h at 37°C in the presence of 2-mercaptoethanol just before use. This increased inhibition is consistent with previous observations with a lysate of rabbit reticulocytes and is probably related to the splitting of the modeccin molecule into two subunits on reduction (Gasperi-Campani *et al.*, 1978). However, further experiments in the present paper were performed with undissociated modeccin, which is more stable in solution.

Since in the system of Fig. 1 protein synthesis starts from phenylalanyl-tRNA, the activation of amino acids can be ruled out as the site of action of modeccin. Possible targets are either ribosomes or soluble factors.

Table 1 shows the effect of modeccin when non-washed ribosomes were preincubated with the toxin, centrifuged and tested for phenylalanine polymer-

Fig. 1. Effect of modeccin on poly(U)-directed [¹⁴C]phenylalanine polymerization The reaction was carried out as described in the Experimental section; 10.88 pmol of KCl-washed ribosomes was present in (a) and 11.06 pmol of non-washed ribosomes in (b). ●, Control; ○, modeccin; △, modeccin preincubated for 1 h at 37°C in the presence of 1% 2-mercaptoethanol.

Table 1. Effect of pretreatment of ribosomes with modeccin on poly(U)-directed [¹⁴C]phenylalanine polymerization Non-washed ribosomes (352 pmol) in 1 ml of medium A were incubated for 30 min at 24°C in the absence (control ribosomes) or in the presence (modeccin-treated ribosomes) of modeccin (19.35 μg). Control and modeccin-treated ribosomes were centrifuged through 1.5 ml of 5% (w/v) sucrose in medium A. The pellets were resuspended in 150 μl of medium A and poly(U)-directed [¹⁴C]phenylalanine polymerization was assayed on samples containing 12 pmol of ribosomes as described in the Experimental section in the absence and in the presence of added modeccin (5 μg).

Ribosomes	Addition to assays	[¹⁴ C]Phenylalanine polymerized (pmol)	Activity (% of control)
Control	None	10.44	100.0
	Modeccin	0.62	5.9
Modeccin-treated	None	0.27	2.6
	Modeccin	0.13	1.3

ization without further addition of modeccin. In spite of the removal of the toxin, ribosomes were over 95% inhibited in protein synthesis, i.e. inhibition was even greater than that observed when 5 μg of modeccin was added to non-treated ribosomes during the assay. Thus ribosomes and not soluble enzymes are the target of modeccin. To ascertain whether modeccin acts only on undissociated ribosomes or also on isolated subunits and eventually on which of them, 40S and 60S subunits were separately incubated in the absence and in the presence of modeccin, centrifuged to remove the inhibitor, and tested for phenylalanine polymerization after reassociation with the complementary

control or treated subunit. Table 2 shows that reassociation of control 60S subunits with modeccin-treated 40S subunits gives fully active ribosomes. This indicates that the isolated small subunit is not affected by the inhibitor, and moreover that the inhibitor is completely removed by the centrifugation procedure. On the contrary, inhibition was substantial with modeccin-treated 60S subunits, both when they were complemented with modeccin-treated or control 40S subunits. Similarly to ricin, modeccin is thus a powerful inhibitor of the 60S ribosomal subunit. As shown in Tables 3 and 4, modeccin also behaves like ricin when tested on the two partial reac-

Table 2. *Effect of pretreatment of ribosomal subunits with modeccin on poly(U)-directed [^{14}C]phenylalanine polymerization*
Isolated 40S (430 pmol) and 60S (446 pmol) subunits were incubated in the absence and in the presence of modeccin (19.35 μg) and centrifuged through 5% sucrose as described for 80S ribosomes in Table 1. Poly(U)-directed [^{14}C]phenylalanine polymerization catalysed by the control and modeccin-treated subunits (12 pmol of 40S and 12 pmol of 60S) was assayed as described in the Experimental section.

Subunits		[^{14}C]Phenylalanine polymerized (pmol)	Activity (% of control)
40S	60S		
Control	Control	9.75	100.0
Modeccin-treated	Control	9.71	99.6
Modeccin-treated	Modeccin-treated	1.35	13.8
Control	Modeccin-treated	1.13	11.6

Table 3. *Puromycin reactivity of N-acetyl[^3H]phenylalanyl-tRNA non-enzymically bound to 80S ribosomes reconstituted from the isolated subunits: effect of modeccin*

The experiment was carried out in three steps. Step 1: 185 pmol of 40S subunits in 250 μl of medium C supplemented with magnesium acetate to a final concentration of 20 mM was incubated for 20 min at 24°C with 500 μg of poly(U) and 249 pmol of N-acetyl[^3H]phenylalanyl-tRNA. At the end of incubation the 40S subunits were centrifuged through 0.85 ml of 6% sucrose in medium C. Step 2: the washed 40S subunits were resuspended in 250 μl of medium C, and bound N-acetyl[^3H]phenylalanyl-tRNA was assayed on a 30 μl sample by the Millipore-filter technique. To two 100 μl samples (each containing 6.3 pmol of bound N-acetyl[^3H]phenylalanyl-tRNA) was added 100 pmol of 60S subunits that had been incubated for 5 min at 24°C in 100 μl of medium C in the absence or in the presence of 6.44 μg of modeccin; incubation was for 15 min at 24°C. Step 3: three 60 μl portions were withdrawn from each sample; one received puromycin (50 μg in 10 μl of water) and two an equal volume of water; after a further 25 min at 24°C, the puromycin-treated sample was analysed for the synthesis of N-acetyl[^3H]phenylalanyl-puromycin; of the two samples that had received water, one was used as blank for the puromycin reaction, and the other was checked for the amount of N-acetyl[^3H]phenylalanyl-tRNA bound at the end of the experiment. The residual 20 μl of the two samples from step 2 were tested for poly(U)-directed phenylalanine polymerization after addition of the appropriate components as described in the Experimental section.

Addition to 60S subunit	N-Acetyl[^3H]phenylalanyl-tRNA bound (pmol)	N-Acetyl[^3H]phenylalanyl-puromycin synthesized (pmol)	[^{14}C]Phenylalanine polymerized (pmol)
None	1.87	1.19	9.24
Modeccin	1.89	1.19	2.77

Table 4. *Effect of modeccin on the EF 2-mediated binding of p[CH $_2$]ppG to KCl-washed ribosomes*

The binding was assayed as described in the Experimental section; KCl-washed ribosomes (27 pmol in 30 μl of medium B) were preincubated for 5 min at 24°C both in the absence and in the presence of 1.29 μg of modeccin before the addition of EF 2 and [^3H]p[CH $_2$]ppG.

	[^3H]p[CH $_2$]ppG (pmol) bound to			EF 2-mediated binding of [^3H]p[CH $_2$]ppG	
	Ribosomes	EF 2	Ribosomes + EF 2	Amount bound (pmol)	Activity (% of control)
Modeccin					
Absent	0.17	5.83	18.92	12.92	100
Present	0.21	6.92	8.81	1.68	13

tions that entirely or largely depend on the larger subunit, i.e. peptide-bond formation and binding of EF 2.

Since *A. salina* ribosomes are naturally devoid of nascent peptides and endogenous mRNA (Hultin & Morris, 1968), peptide-bond formation could not be assayed by measuring the radioactivity that becomes acid-insoluble on interaction of radioactive puromycin with nascent peptides located in the P site

(Montanaro *et al.*, 1973). The reaction (Leder & Bursztyn, 1966) between puromycin and a radioactive N-blocked aminoacyl-tRNA prebound to ribosomes was used instead. As shown in Table 3, at 20 mM-magnesium acetate and in the presence of poly(U), N-acetylphenylalanyl-tRNA binds to a site of the *A. salina* 40S subunit from which it reacts with puromycin on addition of the 60S subunit. The extent of the reaction with puromycin, catalysed by

60S peptidyltransferase, was not affected by treatment of the 60S subunit with modeccin. Phenylalanine polymerization, tested in the same experiment, was inhibited by 70%.

Although peptidyltransferase was unaffected by modeccin, the toxin greatly decreased the EF 2-mediated binding of [^3H]p[CH $_2$]ppG to ribosomes (Table 4). This reaction measures in an indirect way the affinity of ribosomes for EF 2 (Richter, 1973).

Although there is full agreement that ricin and other related plant toxins such as abrin and croton inhibit the binding of EF 2 to ribosomes, their effect on the binding of EF 1 is still a source of controversy. Montanaro *et al.* (1973) and Sperti *et al.* (1975, 1976) found that ricin had little influence on the EF 1- and guanine nucleotide-dependent binding of [^{14}C]phenylalanyl-tRNA to rat liver and to *A. salina* ribosomes, and similar results were obtained by Nolan *et al.* (1976) working with ribosomes from Krebs II mouse ascites cells. On the contrary, Carrasco *et al.* (1975) and Fernandez-Puentes *et al.* (1976a) described experiments in which ricin and abrin strongly inhibited the EF 1- and GTP-dependent binding of [^{14}C]phenylalanyl-tRNA to rabbit reticulocyte ribosomes; they pointed out that the lack of inhibition observed by Montanaro *et al.* (1973) and other workers could depend on either the

small proportion of active ribosomes or the saturating concentrations of EF 1 present in the assays.

Grasmuk *et al.* (1976) have presented evidence that the binding of EF 1 to ribosomes can occur in the absence of GTP and phenylalanyl-tRNA. This binding is particularly substantial with poly(U)-programmed ribosomes precharged with tRNA^{Phe} in their P site; the EF 1 not only catalyses the binding of the first phenylalanyl-tRNA, but remains bound and functionally active on ribosomes during all phases of peptide-chain elongation.

In the present paper the effect of modeccin on the binding of EF 1 has been investigated taking into consideration the above results and observations.

At 20mM-Mg²⁺ and in the presence of poly(U), [^{14}C]phenylalanyl-tRNA binds to ribosomes in a non-enzymic reaction. As shown in Fig. 2(a) (KCl-washed ribosomes), tRNA^{Phe} inhibits this binding, indicating a competition between aminoacylated and deacylated tRNA^{Phe} for the same site on poly(U)-programmed ribosomes. The amount of [^{14}C]phenylalanyl-tRNA bound and the effect of increasing concentrations of tRNA^{Phe} were the same both in the absence and in the presence of modeccin; this indicates that the inhibitor is without effect on the two non-enzymic reactions. Similar results were obtained with non-washed ribosomes (Fig. 2b).

Fig. 2. Poly(U)-directed non-enzymic binding of [^{14}C]phenylalanyl-tRNA to ribosomes: effect of tRNA^{Phe} and of modeccin. KCl-washed ribosomes (a) and non-washed ribosomes (b) (290 pmol in each case) were preincubated 5 min at 24°C both in the absence and in the presence of 12.88 μg of modeccin. The non-enzymic binding of [^{14}C]phenylalanyl-tRNA was carried out on samples containing 20 pmol of ribosomes as described in the Experimental section. The amount of [^{14}C]phenylalanyl-tRNA bound in the absence of poly(U) (2.32 pmol/100 pmol of KCl-washed ribosomes and 6.55 pmol/100 pmol of non-washed ribosomes) was unaffected both by tRNA^{Phe} and by modeccin and was subtracted from all data. ●, Control; ○, modeccin.

Since modeccin does not interfere with the poly(U)-directed binding of tRNA^{Phe}, the effect of the inhibitor on the EF 1-dependent binding of [¹⁴C]phenylalanyl-tRNA could be studied with both uncharged ribosomes and poly(U)-programmed ribosomes charged with tRNA^{Phe} (Fig. 3). In agreement with the results of Grasmuk *et al.* (1976), incubation of ribosomes with tRNA^{Phe} before the addition of EF 1 stimulated the subsequent binding of [¹⁴C]phenylalanyl-tRNA. Modeccin had in all cases some inhibitory effect on the binding reaction, but: (i) inhibition did not depend on the amount of active ribosomes (on the contrary it was often lower with tRNA^{Phe}-stimulated ribosomes with respect to uncharged ribosomes); (ii) inhibition did not decrease when the concentration of EF 1 was increased [on the contrary, maximal inhibition was observed with high concentrations of EF 1 and uncharged ribosomes (Fig. 3*a*)]. As shown in Fig. 3(*b*), the onset of inhibition was time-dependent, in spite of the fact that ribosomes had been preincubated with modeccin before the addition of the components of the binding reaction.

Table 5 compares the effects of modeccin, ricin

and abrin on the EF 1-dependent binding reaction carried out in the conditions that gave maximum inhibition, i.e. high concentration of EF 1 and long incubation time. The effects of the three inhibitors were practically the same.

Since when tested in the partial reactions the interaction of ribosomes with both elongation factors was inhibited, although to a different extent, by modeccin, the effect of the inhibitor on protein synthesis was reinvestigated in an attempt to elucidate which of the two interactions is primarily affected during the repetitive steps of the elongation cycle.

Protein synthesis was carried out both in the absence and in the presence of tRNA^{Phe} and both the total ribosome-bound radioactivity and the hot acid-insoluble radioactivity (long-chain peptides) were measured. The difference gave the amount of [¹⁴C]phenylalanine bound as oligophenylalanyl-tRNA (short-chain peptides) and/or phenylalanyl-tRNA. Table 6 shows the results obtained with KCl-washed and 's-105 supernatant' as source of elongation factors. In both experiments the presence of tRNA^{Phe} alone did not substantially modify the

Fig. 3. EF 1-dependent binding of [¹⁴C]phenylalanyl-tRNA to KCl-washed ribosomes: effect of tRNA^{Phe} and of modeccin. KCl-washed ribosomes (290 pmol) were preincubated for 5 min at 24°C both in the absence and in the presence of 12.88 µg of modeccin. The experiment was then carried out in three steps. Step 1: 16 pmol of control or modeccin-treated ribosomes was incubated for 10 min at 24°C in 50 µl of medium B containing 200 µg of poly(U); 0.06 A₂₆₀ unit of tRNA^{Phe} was present where indicated. Step 2: the volume of the samples was brought to 80 µl by addition of 0.25 M-sucrose/50 mM-Tris/HCl buffer (pH 7.4)/0.1 mM-EDTA/2 mM-dithiothreitol containing variable amounts of EF 1 in (*a*) and 38.2 µg of EF 1 in (*b*); incubation was for 10 min at 24°C. Step 3: 500 nmol of GTP and 25 pmol of [¹⁴C]phenylalanyl-tRNA were added in 30 µl of 130 mM-Tris/HCl buffer (pH 7.4)/200 mM-KCl/12 mM-magnesium acetate/3 mM-dithiothreitol; incubation was at 24°C for 30 min in (*a*) and as indicated in (*b*). The total bound radioactivity was measured. ●—●, Modeccin absent, tRNA^{Phe} absent; ○—○, modeccin present, tRNA^{Phe} absent; ●— — — ●, modeccin absent, tRNA^{Phe} present; ○— — — ○, modeccin present, tRNA^{Phe} present.

Table 5. *Effect of modeccin, ricin and abrin on the EF 1-dependent binding of [¹⁴C]phenylalanyl-tRNA to KCl-washed ribosomes*

The experiment was carried out as described in the legend to Fig. 3. KCl-washed ribosomes (79 pmol) were pre-incubated 5 min at 24°C in the absence and in the presence of the inhibitors (3.22 µg of modeccin, 3.17 µg of ricin and 3.08 µg of abrin); 38.2 µg of EF 1 protein was present in step 2; the incubation of step 3 was for 30 min at 24°C.

[¹⁴C]Phenylalanyl-tRNA bound (pmol/100 pmol of ribosomes)

Inhibitor	tRNA ^{Phe} absent	Activity (% of control)	tRNA ^{Phe} present	Activity (% of control)
None	45.1	100	64.9	100
Modeccin	26.8	59	48.3	74
Ricin	23.3	52	44.5	69
Abrin	26.1	58	44.1	68

Table 6. *Protein synthesis of KCl-washed ribosomes: effect of tRNA^{Phe} and of modeccin on the total binding of [¹⁴C]phenylalanine and on its distribution in long- and short-chain peptides*

KCl-washed ribosomes (120 pmol) were preincubated 5 min at 24°C in the absence or in the presence of modeccin: 6.44 µg in (a) and 12.88 µg in (b). Expt. 1 was carried out in three steps. Step 1: 10 pmol of the control or modeccin-treated ribosomes was incubated for 10 min at 24°C in 50 µl of medium B containing 200 µg of poly(U); 0.06 A₂₆₀ unit of tRNA^{Phe} was present where indicated. Step 2: 15 µl of 's-105 supernatant' (375 µg of protein) was added followed by another 10 min incubation period at 24°C. Step 3: 500 nmol of GTP and 36 pmol of [¹⁴C]phenylalanyl-tRNA were added in 35 µl of 115 mM-Tris/HCl buffer (pH 7.5)/170 mM-KCl/10 mM-magnesium acetate/3 mM-dithiothreitol; incubation was for 30 min at 24°C. Expt. 2: all the above components were added at time zero, and incubation was for 30 min at 24°C. One half of each sample was used for the determination of hot-acid-insoluble radioactivity; on the other half total ribosome-bound radioactivity was measured by the Millipore-filter technique. [¹⁴C]Phenylalanine bound as short-chain peptides and/or phenylalanyl-tRNA was calculated by difference.

[¹⁴C]Phenylalanine bound (pmol/100 pmol of ribosomes)

Expt.	Inhibitor	tRNA ^{Phe}	(a) Total (pmol)	(b) Hot-acid-insoluble (long-chain peptides)		Difference (a - b) (short-chain peptides and/or phenylalanyl-tRNA)	
				(pmol)	(% of control)	(pmol)	(% of control)
1	None	—	156	126	100	30	100
	Modeccin (a)	—	79	57	45	22	73
	Modeccin (b)	—	80	44	35	36	120
	None	+	154	118	100	36	100
	Modeccin (a)	+	147	93	79	54	150
	Modeccin (b)	+	152	80	68	72	200
2	None	—	106	83	100	23	100
	Modeccin (a)	—	32	21	25	11	48
	Modeccin (b)	—	15	7	8	8	35
	None	+	128	110	100	18	100
	Modeccin (a)	+	116	73	66	43	239
	Modeccin (b)	+	119	57	52	62	344

total radioactivity bound to ribosomes, nor its distribution amongst short- and long-chain peptides. A striking effect of tRNA^{Phe} appeared instead in the presence of modeccin. The inhibitory effect of modeccin on the total ribosome-bound radioactivity disappeared and this was because of: (i) a greatly decreased effect of modeccin on the synthesis of long-chain peptides; (ii) a parallel strong increment in the radioactivity bound as phenylalanyl-tRNA or short-chain peptides. These results can be explained only if we assume a synergic action of tRNA^{Phe} and modeccin in engaging a greater number of ribo-

somes in protein synthesis, tRNA^{Phe} stimulating the binding of the first phenylalanyl-tRNA, and modeccin preventing the full elongation of initiated chains.

Results leading to the same conclusions were obtained when protein synthesis was investigated with non-washed ribosomes carrying contaminating elongation factors in the absence of added 's-105 supernatant' (Table 7). In the absence of modeccin and tRNA^{Phe} enzymes were totally engaged in the synthesis of long-chain peptides. In the presence of tRNA^{Phe} the number of ribosomes engaged in protein synthesis increased and short-chain peptides

Table 7. Protein synthesis of non-washed ribosomes in the absence of added elongation factors: effect of tRNA^{Phe} and of modeccin

Non-washed ribosomes (140 pmol) were preincubated 5 min at 24°C both in the absence and in the presence of 6.44 µg of modeccin. The experiment was then carried out as described in Table 6, Expt. 1, except that 's-105 supernatant' was omitted from step 2.

Inhibitor	tRNA ^{Phe}	[¹⁴ C]Phenylalanine bound (pmol/100 pmol of ribosomes)		
		(a) Total	(b) Hot acid-insoluble (long-chain peptides)	Difference (a - b) (short-chain peptides and/or phenylalanyl-tRNA)
None	—	5.81	6.09	—
Modeccin	—	5.28	0.46	4.82
None	+	22.06	15.14	6.92
Modeccin	+	7.44	0.94	6.50

appeared. In this case the most striking effect of modeccin appeared in the absence of tRNA^{Phe}: the synthesis of long-chain peptides was practically suppressed, but the total radioactivity bound to ribosomes was unchanged because the block of elongation made EF 1 available for the binding of [¹⁴C]-phenylalanyl-tRNA to a greater number of ribosomes. In the presence of tRNA^{Phe}, modeccin, in spite of the block of elongation, did not increase the radioactivity bound as short-chain peptides above the value induced by tRNA^{Phe} alone. However, since the method does not discriminate between phenylalanyl-tRNA and oligopeptides of phenylalanine, the lack of increase does not preclude the existence of a greater number of ribosomes carrying phenylalanyl-tRNA; probably their number cannot be further augmented because EF 1 is limiting.

Discussion

Modeccin inhibits polypeptide-chain elongation in a cell-free system by a mechanism very similar to that of ricin, since: (i) it inactivates the 60S ribosomal subunit; (ii) it does not affect peptide-bond formation, measured by the puromycin reaction, thus indicating that the lesion induced in the 60S subunit is at a site distinct from the peptidyltransferase centre; (iii) it strongly inhibits the EF 2-dependent binding of p[CH₂]ppG to ribosomes, indicating that some site of the large subunit involved in the binding of EF 2 is damaged.

As reported above, the study of the effect of ricin and other related plant toxins on the EF 1-dependent binding of aminoacyl-tRNA to ribosomes has given contradictory results (Montanaro *et al.*, 1973; Sperti *et al.*, 1975, 1976; Nolan *et al.*, 1976; Carrasco *et al.*, 1975; Fernandez-Puentes *et al.*, 1976a). In a previous paper from this laboratory (Sperti *et al.*, 1976) it was stated that ricin scarcely affected the EF 1-dependent binding of phenylalanyl-tRNA to *A. salina* ribosomes. KCl-washed ribosomes were prepared as described by Zasloff & Ochoa (1971) and the binding

assay was preceded by preincubation of ribosomes with diphtheria toxin and NAD⁺; this was to inactivate by ADP-ribosylation the small amounts of EF 2 that were present in the ribosomal preparations and that gave substantial elongation on addition of EF 1. In the experiments reported in the present paper the ADP-ribosylation step was omitted, since ribosomes were prepared by the method of Sierra *et al.* (1974), in which a drastic salt-washing procedure greatly decreases the contamination with elongation factors. Inhibition of the binding reaction was observed in the presence of both ricin and modeccin (Fig. 3 and Table 5). However, in the absence of inhibitors, small amounts of hot acid-insoluble radioactivity (2 pmol/100 pmol of ribosomes in the experiment of Fig. 3b) appeared at the end of the binding assay, i.e. after 30 min of incubation. This indicates that trace amounts of contaminating EF 2 also remain in *A. salina* ribosomes washed as described by Sierra *et al.* (1974). If these traces, although not detectable by ADP-ribosylation, were able, on addition of EF 1, to promote the formation of some polyphenylalanine measurable as hot acid-insoluble radioactivity, there is good reason to suspect the formation of even greater amounts of oligopeptides of phenylalanine, not measurable as hot acid-insoluble radioactivity, but retained on Millipore filters and counted as total bound radioactivity. The effect of the presence of these oligopeptides will be an overestimate of the number of control ribosomes carrying phenylalanyl-tRNA, and inhibition of their formation by inhibitors of elongation will simulate an inhibition of the binding reaction.

The conclusion that the inhibition of the enzymic binding of aminoacyl-tRNA is, at least in the experimental conditions reported in the present paper, mostly apparent is supported by the following observations: (i) inhibition did not appear at short times of incubation, i.e. before the onset of elongation; (ii) the presence of tRNA^{Phe}, which stimulated the binding reaction, decreased rather than increased the inhibition.

The analysis of the distribution of ribosome-bound radioactivity amongst long- and short-chain peptides during protein synthesis supports the view that the binding of the first aminoacyl-tRNA to ribosomes is not affected by modeccin. In fact (i) the presence of modeccin alone decreased the total radioactivity bound to KCl-washed ribosomes, mostly by inhibiting the synthesis of long chains (Table 6); (ii) modeccin added together with tRNA^{Phe} to KCl-washed ribosomes still inhibited the synthesis of long chains; the formation of short chains was on the contrary stimulated (Table 6), probably because the block of elongation made available a greater amount of [¹⁴C]phenylalanyl-tRNA for its binding to tRNA^{Phe}-activated ribosomes; (iii) with non-washed ribosomes modeccin again suppressed the synthesis of long-chain peptides catalysed by endogenous elongation factors, but increased the radioactivity bound as short chains, probably because the block of elongation made limiting EF 1 available for the binding of [¹⁴C]phenylalanyl-tRNA to new ribosomes.

All the above results clearly indicate that the step inhibited by modeccin during the complete elongation cycle is beyond the binding of the first aminoacyl-tRNA. However, whether inhibition involves only the EF 2-catalysed reactions or also the EF 1-catalysed binding of phenylalanyl-tRNA to ribosomes carrying initiated chains remains an open question.

The data of Fig. 1 indicate that the non-washed ribosomes used in the present experiments were more susceptible than the KCl-washed ribosomes to the action of modeccin. With other ribosomal preparations washed and non-washed ribosomes behaved in the same way with respect to the inhibitor. Fernandez-Puentes *et al.* (1976b) reported that added EF 2 in the presence of GTP strongly protects ribosomes from the inactivation by abrin and ricin. If modeccin acts as ricin and abrin it must be concluded that the presence of endogenous elongation factors does not protect ribosomes from the action of the inhibitors.

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