Inhibition by Ricin of Protein Synthesis *in vitro*

RIBOSOMES AS THE TARGET OF THE TOXIN

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1. Ricin (a toxic protein from the seeds of *Ricinus communis*) is a powerful inhibitor of the poly(U)-directed incorporation of phenylalanine into polypeptides catalysed by isolated rat liver ribosomes and elongation factors 1 and 2 (EF 1 and EF 2). The inhibition can be largely overcome by increasing the concentration of ribosomes. 2. The toxin does not affect the binding of phenylalanyl-tRNA to ribosomes catalysed by EF 1, nor does it inhibit the puromycin reaction used as a test for peptide-bond formation catalysed by ribosomes. 3. Ricin inhibits the ribosome-linked GTP hydrolysis catalysed by EF 2. 4. Ribosomes treated with ricin and washed through sucrose gradients containing 0.6 M-NH₄Cl are functionally inactive in those assay systems that are sensitive to the presence of added toxin. 5. It is suggested that ricin brings about an irreversible modification of ribosomes which impairs their ability to interact with EF 2. Since ricin inhibits at a molar concentration much lower than that of ribosomes it probably acts catalytically. No added cofactor is necessary for the inhibitory action of the toxin.

Ricin, a protein from castor beans (the seeds of *Ricinus communis*), is extremely toxic to animals and causes necrotic lesions in the gastrointestinal system, in the spleen and lymph nodes, and in the liver (Waller *et al.*, 1966).

It has been reported that ricin has an inhibitory effect on the growth of Ehrlich ascites tumour (Lin *et al.*, 1970) and brings about inhibition of protein synthesis in Ehrlich-ascites-tumour cells *in vitro* and in rat liver *in vivo*, affecting DNA synthesis only slightly and RNA synthesis not at all (Lin *et al.*, 1971). Olsnes & Pihl (1972a) reported that ricin inhibits protein synthesis in a cell-free system prepared from rabbit reticulocytes, and suggested that this effect was due to inhibition of the elongation of polypeptide chains, the initiation being unaffected.

The present experiments were undertaken with the aim of clarifying the mechanism whereby ricin affects protein synthesis. With the use of a reconstituted system of rat liver ribosomes and EF 1* and EF 2 evidence was obtained (i) that ricin inhibits the elongation of polypeptide chains by interfering with the translocation reaction catalysed by EF 2, without affecting the binding of aminoacyl-tRNA, and (ii)

that this is due to damage to the ribosomes, EF 2 appearing unaffected.

Experimental

Materials

Ricin was purified as described by Moulé (1951). Its LD₅₀, tested in Wistar albino rats, was 7.5 µg/kg body wt. given intraperitoneally. Its molecular weight was assumed to be 65000 (Lin *et al.*, 1970). Diphtheria toxin, purified by the method of Pope & Stevens (1958), was obtained from the Istituto Sieroterapico e Vaccinogeno Toscano 'Sclavo' (Siena, Italy).

All the chemicals used were analytical grade. Norit A (activated charcoal), NAD⁺ and GTP were products of Sigma Chemical Co., St. Louis, Mo., U.S.A. Puromycin dihydrochloride was from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. Poly(U) (potassium salt) was from C. F. Boehringer and Soehne G.m.b.H., Mannheim, Germany. [γ-³²P]-GTP (509 mCi/mmol), [8-³H]GTP (12 Ci/mmol) and [8-³H]puromycin (2 Ci/mmol) were from The Radiochemical Centre, Amersham, Bucks., U.K. [adenosine-³H]NAD⁺ (1.13 Ci/mmol) and L-[¹⁴C]phenylalanyl-tRNA (0.137 µCi of [¹⁴C]phenylalanine/mg of tRNA; 385 mCi/mmol of L-phenylalanine) were

* Abbreviations: EF 1, elongation factor 1; EF 2, elongation factor 2; A site, aminoacyl-tRNA site; P site, peptidyl-tRNA site; GTPase, guanosine triphosphatase.

obtained from New England Nuclear Corp., Boston, Mass., U.S.A.

Preparation of ribosomes

Rat liver polyribosomes were prepared as described by Staehelin & Falvey (1971) and stored at -20°C in small portions containing 150–300 E_{260} units/ml.

Puromycin-treated ribosomes. Ribosomes freed of endogenous peptidyl-tRNA were prepared by incubating polyribosomes at a final concentration of 20 E_{260} units/ml in a complete protein-synthesizing system (Staehelin & Falvey, 1971) containing 50 μg of puromycin/ml. After 50 min at 35°C , the mixture was chilled in ice; KCl and NH_4Cl were added to final concentrations of 0.25 M and 0.5 M respectively and 9 ml samples were layered over discontinuous sucrose gradients consisting of 9 ml of 2 M-sucrose and 9 ml of 0.7 M-sucrose, both containing 20 mM-Tris-HCl buffer, pH 7.5, 3 mM-magnesium acetate and 1 mM-dithiothreitol; 0.6 M- NH_4Cl was present in the 0.7 M-sucrose layer. The gradients were centrifuged for 24 h at 4°C in a Spinco 30 Angle rotor (Beckman model L2-65K ultracentrifuge) at 78000 g_{av} . Two further washings on the same discontinuous sucrose gradients were performed to remove all adherent EF 2 and GTP from ribosomes.

Translocated ribosomes. Ribosomes with their nascent peptidyl-tRNA at the P site were prepared as described by Baliga & Munro (1972) by incubating polyribosomes at a final concentration of 3 E_{260} units/ml in 54 ml of a reaction medium containing 0.5 mM-GTP and 24 μg of EF 2 protein/ml measured by the method of Lowry *et al.* (1951) with bovine serum albumin as standard. The ionic conditions were 150 mM- NH_4Cl , 7.5 mM-magnesium acetate, 20 mM-Tris-HCl buffer, pH 7.5, 1 mM-dithiothreitol. After 10 min at 37°C , the ribosomes were spun down and then washed twice on discontinuous sucrose gradients containing 0.6 M- NH_4Cl , as for puromycin-treated ribosomes.

Non-translocated ribosomes. Control ribosomes were incubated under the same ionic conditions as translocated ribosomes, but in the absence of EF 2 and GTP. They were then washed through the same sucrose gradients.

Ricin-treated ribosomes. The incubation mixtures for the preparation of translocated and non-translocated ribosomes contained ricin at a final concentration of 2 μg /ml. After 10 min at 37°C , ribosomes were spun down and washed twice through the discontinuous sucrose gradients containing NH_4Cl .

All ribosomal pellets were resuspended in 0.25 M-sucrose and ribosome concentration was calculated from the E_{260} , assuming $E_{1\text{cm}}^{1\%}$ to be 145 and the molecular weight of the ribosome to be 4.1×10^6 (Baliga & Munro, 1972). With the latter value, 1 mg of ribosomes corresponds approximately to 250 pmol.

Other methods

Preparation of elongation factors. EF 1 and EF 2 were obtained from rat liver 'pH 5 supernatant' as described by Moldave *et al.* (1971). After adsorption on hydroxyapatite and stepwise elution with 0.125 M-, 0.175 M- and 0.250 M-potassium phosphate buffer, pH 6.8, the EF 2 present in the 0.125 M-phosphate eluate and the EF 1 present in the 0.250 M-phosphate eluate were further purified by separate chromatography on DEAE-cellulose as described by Felicetti & Lipmann (1968). The protein content of the enzyme preparations was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Poly(U)-directed incorporation of phenylalanine into polypeptide. The reaction medium used contained in a final volume of 0.25 ml: 150 mM- NH_4Cl , 9 mM-magnesium acetate, 20 mM-Tris-HCl buffer, pH 7.5, 1 mM-dithiothreitol, 2 mM-GTP, 73 μg of [^{14}C]phenylalanyl-tRNA and 200 μg of poly(U). Ribosomes and the EF 1 and EF 2 preparations were present in the amounts indicated in the legends to Tables. After 10 min at 37°C , 0.25 ml of 10% (w/v) trichloroacetic acid was added and the samples were heated at 90°C for 10 min. The hot-acid-insoluble radioactivity was collected on glass-fibre filters (Whatman GF/C), washed with 5% trichloroacetic acid and measured.

Phenylalanyl-tRNA binding to ribosomes. Ribosomes (500 μg), 146 μg of [^{14}C]phenylalanyl-tRNA and 200 μg of poly(U) were incubated at 37°C for 10 min in 0.5 ml of 20 mM-Tris-HCl buffer, pH 7.5, containing 150 mM- NH_4Cl , 9 mM-magnesium acetate, 1 mM-dithiothreitol and 2 mM-GTP. Most of the samples also contained 0.5 mM-puromycin (Ibuki & Moldave, 1968). Incubations were carried out in the presence and in the absence of 30 μg of EF 1 protein. At the end of incubation, the reaction mixtures were diluted with 5 ml of ice-cold 20 mM-Tris-HCl buffer, pH 7.5, containing 150 mM- NH_4Cl , 9 mM-magnesium acetate and 1 mM-dithiothreitol and filtered immediately on Millipore filters (HA; average pore size 0.45 μm). The filters were washed three times with 5 ml of cold buffer, dried and assayed for radioactivity.

Peptidyl- $[\text{}^3\text{H}]$ puromycin reaction. Ribosomes (250 μg) were allowed to react with puromycin dihydrochloride in a mixture containing, in 0.5 ml, 60 mM-Tris-HCl buffer, pH 7.5, 12 mM- MgCl_2 , 300 mM- NH_4Cl and 10 μM - $[\text{}^3\text{H}]$ puromycin. After 15 min of incubation at 37°C the samples were analysed for hot-acid-insoluble radioactivity (Skogerson & Moldave, 1968).

GTPase assay. GTPase activity was assayed as described by Felicetti & Lipmann (1968) in incubation mixtures containing, in 0.125 ml, 50 mM-Tris-HCl buffer, pH 7.5, 80 mM- NH_4Cl , 10 mM- MgCl_2 , 16 mM-dithiothreitol and 5 nmol of $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ (diluted with unlabelled GTP to a specific radioactivity of 50 d.p.m./pmol); 200 μg of NH_4Cl -washed

ribosomes and 24 μg of EF 2 protein were present where indicated. After 15 min at 37°C, the reaction was terminated by the addition of 1 ml of ice-cold 2.6% (w/v) trichloroacetic acid containing 1 mM- KH_2PO_4 and 30 mg of Norit A. The mixture was shaken for 30 s on a Vortex mixer and centrifuged for 5 min at 2500 g in a Martin Christ Universal centrifuge at 2°C. Supernatants were filtered through Whatman no. 1 paper and 0.1 ml samples of the filtrates were assayed for radioactivity. Ribosome-linked GTPase activity was calculated by subtracting the activities of ribosomes alone and of EF 2 alone from the total activity observed when the two components were incubated together.

Binding of [^3H]GTP to EF 2. The effect of ricin on the binding of GTP to EF 2 was studied by equilibrium-dialysis experiments performed as described by Sperti *et al.* (1971). One set of chambers contained EF 2 (624 μg of protein/ml) in one compartment and [^3H]GTP at the same initial concentration (1–18 nM) in both compartments. The second set contained, besides EF 2 and GTP, ricin (20 $\mu\text{g}/\text{ml}$) in both compartments. After 24 h dialysis at 5°C, 0.1 ml samples were withdrawn from both compartments and assayed for radioactivity. The plot of the reciprocal of bound (b) against the reciprocal of free (c) [^3H]GTP concentration according to the equation (Sperti *et al.*, 1971):

$$1/b = 1/np + K_d/np c$$

gave a straight line, from which the concentration of the enzyme-binding sites (np , where n is the stoichiometry and p the molar concentration of the interacting protein) and the dissociation constant (K_d) of the complex of EF 2 with GTP could be calculated.

[^3H]ADP-ribosylation. ADP-ribosylation of EF 2 was performed by allowing the EF 2 preparation to react with [^3H]adenosine-3'NAD $^+$ and diphtheria toxin as described by Raeburn *et al.* (1971). The concentration of EF 2 in the enzyme preparations was calculated from the specific radioactivity of the [^3H]NAD $^+$ employed.

Radioactivity measurements. Dried filters or aqueous samples were put in scintillation vials containing 6 ml of ethylene 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] and were counted for radioactivity in a Nuclear-Chicago mark I scintillation counter (counting efficiency approx. 60% for ^{14}C , 15% for ^3H and 40% for ^{32}P). The counts were corrected to 100% efficiency.

Results

Poly(U)-directed phenylalanine incorporation into polypeptide was severely inhibited in the presence of ricin. In the complete system containing the two elongation factors and 30 pmol of non-translocated ribo-

somes, the degree of inhibition was 33% in the presence of 0.2 μg of ricin/ml, 73% in the presence of 2 μg of ricin/ml and 80% in the presence of 20 μg of ricin/ml. Similarly, with puromycin-treated ribosomes inhibition was 35% in the presence of 0.2 μg of ricin/ml, 72% in the presence of 2 μg of ricin/ml and 82% in the presence of 20 μg of ricin/ml. These inhibitions are calculated from the data reported in Fig. 1. The lower curve in Fig. 1 shows, at the same concentrations of ricin, the incorporation of [^{14}C]phenylalanine on addition of EF 1 alone to ribosomes washed through sucrose gradients lacking NH_4Cl . The incorporation of [^{14}C]phenylalanine depended on endogenous EF 2 adherent to ribosomes. In these conditions the inhibition of phenylalanine incorporation was less severe at the lower concentrations of toxin, as if more ricin were needed to interfere with the ribosome activity catalysed by endogenous EF 2.

To identify in the complete system the component(s) sensitive to the toxin the inhibition of phenylalanine polymerization induced by ricin was measured in experiments in which the concentrations of ribosomes and of the enzymes were varied independently. Table 1 shows the inhibition brought about

Fig. 1. Effect of ricin on poly(U)-directed phenylalanine incorporation into polypeptide

The otherwise standard incubation mixture contained (in 0.25 ml): upper curve, 15 μg of EF 1 protein, 50 μg of EF 2 protein and 30 pmol of NH_4Cl -washed non-translocated (○) or puromycin-treated ribosomes (●); lower curve, 15 μg of EF 1 protein and 30 pmol of ribosomes washed through sucrose gradients without NH_4Cl (Δ).

by ricin (0.8 $\mu\text{g/ml}$) in the presence of a constant concentration of EF 1 and at two different concentrations of EF 2 and of non-translocated ribosomes. The extent of the inhibition was practically the same at two concentrations of EF 2 differing by a factor of 5. On the other hand, the inhibition, which was almost complete at the lower concentration of ribosomes, was decreased to less than 30% by a 2.5-fold increase in ribosome concentration. Since inhibition could be overcome by increasing the concentration of ribosomes, these appeared to be the main target of the toxin in the phenylalanine-incorporating system.

A direct effect of ricin on ribosomes was clearly indicated in experiments in which ribosomes were preincubated with the toxin, washed, and then tested for phenylalanine incorporation without further

addition of ricin. Table 2 shows the activity of translocated and non-translocated ribosomes isolated as described in the Experimental section from preincubation mixtures in the absence or in the presence of ricin (2 $\mu\text{g/ml}$). The addition of EF 1 to once-washed non-translocated ribosomes (EF 2 absent from the preincubation mixture) induced a slight incorporation of [^{14}C]phenylalanine, which increased 10-fold on addition of EF 2. When ricin was present during preincubation, the incorporation in the presence of EF 1 alone was decreased only slightly, whereas the increment due to EF 2 was completely abolished. A second washing of the ribosomes did not modify the results. With translocated ribosomes (EF 2 and GTP present during preincubation) one NH_4Cl washing was insufficient to remove the EF 2

Table 1. *Effectiveness of ricin as inhibitor of phenylalanine polymerization catalysed by low and high concentrations of ribosomes and EF 2*

The otherwise standard assay mixture (see the Experimental section) contained (in 0.25ml): 90 μg of EF 1 protein and two different concentrations of ribosomes and EF 2 as indicated.

Content of ribosomes	Content of EF 2 protein	Radioactivity incorporated (d.p.m.)		Inhibition (%)
		Ricin absent from assay mixture	Ricin present (0.8 $\mu\text{g/ml}$) in assay mixture	
Low (110 μg ; 27.5 pmol)	Low (24 μg)	960	94	90
	High (120 μg)	6694	1329	80
High (275 μg ; 68.75 pmol)	Low (24 μg)	3071	2254	27
	High (120 μg)	9302	7293	22

Table 2. *Effect of preincubation of ribosomes with ricin on [^{14}C]phenylalanine incorporation catalysed by EF 1 and EF 2*

Ribosomes sedimented from the various preincubation mixtures were washed twice with 0.6M- NH_4Cl as described in the Experimental section (see under 'Preparation of ribosomes'). Then 200 μg (50 pmol) of the once-washed ribosomes and 200 μg (50 pmol) of the twice-washed ribosomes were tested under the reaction conditions described in the Experimental section (see under 'Poly(U)-directed incorporation of phenylalanine into polypeptide'). Where indicated, 45 μg of EF 1 and 48 μg of EF 2 protein were added.

Ribosome preparation	Preincubation additions	Number of 0.6M- NH_4Cl washings	Radioactivity incorporated (d.p.m.)		
			No enzyme added	EF 1 added	EF 1 + EF 2 added
Non-translocated ribosomes	None	One	83	968	9131
		Two	89	977	9751
	Ricin	One	87	611	586
		Two	85	522	560
Translocated ribosomes	EF 2, GTP	One	93	3499	9772
		Two	76	993	8870
	EF 2, GTP, ricin	One	81	1274	1023
		Two	89	1155	989

that had adhered to ribosomes during preincubation. In fact the addition of EF 1 alone caused a significant incorporation of [^{14}C]phenylalanine, which increased threefold on addition of EF 2. When ricin was present the binding of EF 2 to ribosomes during preincubation appeared decreased, and, as with non-translocated ribosomes, further addition of EF 2 was completely ineffective on [^{14}C]phenylalanine incorporation. After the second NH_4Cl washing the incorporations with translocated ribosomes approached those observed with non-translocated ribosomes. Free ricin from the preincubation mixtures did not co-sediment with ribosomes through the NH_4Cl -washing procedure, since the addition of ricin-treated ribosomes to control ribosomes did not affect their phenylalanine-polymerizing activity.

Table 3 shows the effect of ricin on the binding of [^{14}C]phenylalanyl-tRNA to ribosomes catalysed by EF 1. The data were obtained after subtraction of two blanks, the total radioactivity bound to ribosomes in the absence of EF 1 and the hot-acid-insoluble radioactivity recovered from duplicate samples incu-

bated in the presence of EF 1. The latter value, which indicates the extent of [^{14}C]phenylalanine polymerization in the presence of EF 1, was negligible when puromycin-treated ribosomes were used or when 0.5 mM-puromycin was present during the binding assay (Ibuki & Moldave, 1968). The data reported in Table 3 clearly indicate that the presence of ricin in the incubation medium did not affect the binding of phenylalanyl-tRNA to puromycin-treated ribosomes (Expt. 1); similarly, the binding activity of ribosomes isolated from preincubation mixtures containing ricin was scarcely affected as compared with that of ribosomes preincubated in the absence of ricin (Expt. 2).

Table 4 shows the effect of ricin on the peptidyl- $[\text{^3H}]$ puromycin reaction catalysed by translocated and non-translocated ribosomes. In the absence of ricin the difference in the amount of $[\text{^3H}]$ puromycin incorporated by translocated and non-translocated ribosomes indicates that 40% of the total peptidyl-tRNA had translocated from the A site to the P site of ribosomes during preincubation with EF 2 and GTP. The presence of ricin during preincubation did

Table 3. *Effect of ricin on the EF 1-catalysed binding of [^{14}C]phenylalanyl-tRNA to ribosomes*

In Expt. 1, 500 μg (125 pmol) of puromycin-treated ribosomes (see the Experimental section) was tested for [^{14}C]phenylalanyl-tRNA binding in the absence and in the presence of different concentrations of ricin added to 0.5 ml of the incubation medium described in the Experimental section. In Expt. 2, 500 μg (125 pmol) of NH_4Cl -washed ribosomes isolated from preincubation mixtures carried out in the absence (control) or in the presence (ricin-treated) of ricin (2 $\mu\text{g}/\text{ml}$) was tested for [^{14}C]phenylalanyl-tRNA binding as described in the Experimental section; 0.5 mM-puromycin was present in the incubation medium. The radioactivity bound in the absence of EF 1, determined as a routine, was less than 30% of the total bound radioactivity; the EF 1-catalysed binding was calculated by difference.

Expt.	Ribosomes	Additions to incubation medium	Radioactivity bound (d.p.m.)
1	Puromycin-treated	None	1062
		Ricin (0.2 $\mu\text{g}/\text{ml}$)	1082
		Ricin (2.0 $\mu\text{g}/\text{ml}$)	1040
		Ricin (20.0 $\mu\text{g}/\text{ml}$)	931
2	Control	Puromycin	976
	Ricin-treated	Puromycin	892

Table 4. *Effect of ricin on the peptidyl- $[\text{^3H}]$ puromycin reaction*

Ribosomes (250 μg , 62.5 pmol) isolated from the various preincubation mixtures were tested in 0.5 ml of the peptidyl- $[\text{^3H}]$ puromycin assay mixture described in the Experimental section.

Ribosome preparation	Preincubation additions	$[\text{^3H}]$ Puromycin reacted (pmol/pmol of ribosomes)	
		Ricin absent from assay mixture	Ricin present (20 $\mu\text{g}/\text{ml}$) in assay mixture
Non-translocated ribosomes	None	0.12	0.13
	Ricin	0.13	0.17
Translocated ribosomes	EF 2, GTP	0.20	0.21
	EF 2, GTP, ricin	0.20	0.25

Table 5. *Effect of ricin on the ribosome-linked GTPase activity of EF 2*

NH₄Cl-washed ribosomes (200 µg, 125 pmol) from preincubations in the absence (control) or in the presence (ricin-treated) of ricin (2 µg/ml) were tested in 0.125 ml of the GTPase assay system as described in the Experimental section.

	Incubation system		[γ - ³² P]GTP hydrolysed (pmol)	Ribosome-linked GTPase activity of EF 2	
	Complementary factors	Ricin (20 µg/ml)		(pmol of [γ - ³² P]GTP hydrolysed)	Activity (%)
Control ribosomes	Ribosomes	—	208	810	100
	EF 2	—	240		
	Ribosomes + EF 2	—	1258		
Control ribosomes	Ribosomes	+	195	349	43
	EF 2	+	218		
	Ribosomes + EF 2	+	762		
Ricin-treated ribosomes	Ribosomes	—	208	342	42
	EF 2	—	230		
	Ribosomes + EF 2	—	780		
Ricin-treated ribosomes	Ribosomes	+	210	310	38
	EF 2	+	200		
	Ribosomes + EF 2	+	720		

not affect this single translocation. A value of 20% for ribosomes reactive with puromycin after translocation is a common finding reported by several authors (Alberghina *et al.*, 1971; Baliga & Munro, 1972; Schneider & Maxwell, 1973). The data of Table 4 show also that the presence of ricin in the peptidyl-^{[3}H]puromycin reaction mixture did not decrease the amount of puromycin-peptide formed. If the puromycin reaction is to be considered an assay for normal peptide formation, we must conclude that transpeptidation is not affected by ricin.

Since ricin did not affect the ability of ribosomes either to bind phenylalanyl-tRNA or to catalyse the peptidyl-puromycin reaction, attention was focused on the interaction between ribosomes and EF 2. Table 5 shows the effect of ricin on the ribosome-linked GTPase activity of EF 2, obtained by subtracting the activities of ribosomes alone and of EF 2 alone from the activity observed when the two components were incubated together. Ricin did not modify the amount of GTP hydrolysed either by ribosomes or by EF 2 tested separately, but considerably decreased the increment observed when the two components were incubated together. The residual activity was 43% when ricin (20 µg/ml) was present in the assay system, and 42% when ribosomes isolated from preincubation mixtures containing 2 µg of ricin/ml were tested. The further addition of ricin to ricin-treated ribosomes hardly modified this value.

The fact that ricin did not modify the amount of GTP hydrolysed by EF 2 alone is consistent with the results of equilibrium-dialysis experiments performed

to study the effect of ricin (20 µg/ml) on the binding of [³H]GTP to EF 2. Although ricin was added in a fivefold molar excess with respect to EF 2 (as indicated by the value for the molarity of binding sites of EF 2 calculated from the ADP-ribosylation test), no modification of the dissociation constant of the EF 2-GTP complex ($K_d = 1 \times 10^{-7}$ M) nor of the molarity of the binding sites of the enzyme for GTP ($np = 7 \times 10^{-8}$ M) was observed.

Discussion

Ricin is a very powerful inhibitor of the poly(U)-directed phenylalanine incorporation catalysed by isolated rat liver ribosomes and EF 1 and EF 2. This is consistent with the results of Olsnes & Pihl (1972a), who obtained inhibition both of endogenous protein synthesis and of the translation of added messenger poly(U) in a system consisting of a lysate from rabbit reticulocytes. The data of Olsnes & Pihl (1972a) indicated that the toxin interfered with some component of the cell-free system necessary for the elongation of already initiated polypeptide chains.

Polypeptide-chain elongation, i.e. the translation of the internal codons of mRNA concomitant to the stepwise addition of amino acids from aminoacyl-tRNA molecules to the growing polypeptide chain, is a repetitive phenomenon which occurs through a cyclic series of reactions (Moldave, 1972). Each cycle can be conveniently divided into three steps: (i) the binding of the appropriate aminoacyl-tRNA at the A site of the ribosome, catalysed by EF 1; (ii) peptide-bond formation at the A site between the

newly bound aminoacyl-tRNA and the peptidyl-tRNA present on the P site, catalysed by ribosomal peptidyltransferase; (iii) translocation of the elongated peptidyl-tRNA from the A site back to the P site, catalysed by EF 2.

When these three steps were analysed separately, ricin did not affect the binding of aminoacyl-tRNA to ribosomes (Table 3), nor did it inhibit peptide-bond formation as assayed by the peptidyl-puromycin reaction (Table 4). The extent of the latter reaction depends on the number of ribosomes carrying peptidyl-tRNA on the puromycin-reactive P site. When ribosomes are preincubated with EF 2 and GTP, translocation of peptidyl-tRNA from the A to the P site occurs, hence increasing the extent of the peptidyl-puromycin reaction. Table 4 shows that the presence of ricin during the preincubation with EF 2 and GTP did not affect this single translocation.

On the other hand, when EF 2-catalysed translocation is repetitively involved in the elongation of polypeptide chains, it is severely inhibited by ricin. This is due to damage to ribosomes and not to EF 2, since (i) the extent of inhibition can be decreased by increasing the amount of ribosomes (Table 1) and (ii) inhibition does occur in the absence of ricin with ribosomes pretreated with the toxin (Table 2).

Under the action of ricin ribosomes seem to become unable to interact with EF 2 as indicated by the inhibition of the ribosome-linked GTPase activity of EF 2 measured uncoupled from translocation (Table 5).

Olsnes & Pihl (1972a) suggested that the inhibitory effect of ricin on elongation is similar to that of diphtheria toxin, although the two toxins act by different mechanisms. Our results indicate that ricin, like diphtheria toxin, inhibits elongation by interfering with the functional interaction of EF 2 with ribosomes. However, diphtheria toxin in the presence of NAD^+ inactivates EF 2 (Collier, 1967; Honjo *et al.*, 1968), whereas ricin modifies ribosomes and makes them unreactive with added EF 2.

The results of the experiments with a low concentration of ribosomes (Table 1) show that an almost complete inhibition of phenylalanine incorporation is obtained at a molar ratio of ricin to ribosomes of 1:10. This ratio appears too low to explain the inhibition on the basis of an irreversible binding of ricin to ribosomes and suggests that ricin may act in a catalytic manner, bringing about an irreversible modification of ribosomes. Olsnes & Pihl (1972a) suggested

that the toxin may act as an enzyme, and showed that inhibition of protein synthesis occurred at a concentration of ricin much lower than that of ribosomes (Olsnes & Pihl, 1972b). It is noteworthy that whatever the mechanism of ricin action may be, it does not require any added cofactor.

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