Inhibition of Protein Synthesis *in vitro* by Crotons and Ricin

EFFECT ON THE STEPS OF PEPTIDE CHAIN ELONGATION

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The effects of crotin I and crotin II on the partial reactions of polypeptide chain elongation were investigated and compared with the known effects of ricin. Crotin II was a more powerful inhibitor than crotin I, but no qualitative differences between the two crotons were found. Rat liver ribosomes, preincubated with crotons and washed through sucrose gradients, remained inactive in protein synthesis. Among the individual steps of elongation, the peptidyltransferase reaction was unaffected by crotons, but some of the reactions that involve the interaction of elongation factors 1 and 2 with ribosomes were modified. A strong inhibition of the binding of elongation factor 2 to ribosomes and a stimulation of the elongation factor 2-dependent GTP hydrolysis were observed; this indicates the formation of a very unstable elongation factor 2-GDP-ribosome complex, which, however, allows a single round of translocation to take place in the presence of elongation factor 2 and added GTP. The elongation factor 1-dependent GTP hydrolysis was inhibited by crotons, whereas the enzymic binding of aminoacyl-tRNA, to both rat liver and *Artemia salina* ribosomes, was scarcely affected. In a protein-synthesizing system the inhibition by crotons and by ricin leads to a block of the nascent peptides on the ribosomal aminoacyl-tRNA site, an effect consistent with inhibition at the level of translocation. The mechanism of action of crotons appears to be very similar to that of ricin.

Among plant toxins, ricin (from *Ricinus communis*), abrin (from *Abrus precatorius*) and crotin (from *Croton tiglium*) were associated in the early literature because of the similarity of the lesions produced in animals poisoned by them (Nicolle & Cesari, 1913). Stirpe *et al.* (1976) have shown that extracts from *Croton tiglium*, like ricin and abrin (Olsnes & Pihl, 1972), inhibit protein synthesis in cell-free systems from rabbit reticulocytes. By chromatography on Sephadex G-100 crude crotin was separated into two toxic fractions. The peak eluted first (crotin I) showed the greatest toxicity *in vivo*. The second peak (crotin II) had a greater effect *in vitro*, inhibition of protein synthesis being of the same order as that obtained with pure ricin, although crotin II was 1000 times less toxic than ricin *in vivo*.

In the present work the action of the two toxic peaks from *Croton tiglium* extracts on the individual steps of polypeptide chain elongation has been investigated; at the same time some of the effects of ricin (Montanaro *et al.*, 1973; Olsnes *et al.*, 1973; Montanaro *et al.*, 1975; Sperti *et al.*, 1975; Carrasco *et al.*, 1975) have been reinvestigated and compared with those of crotin.

Experimental

Materials

Ricin was prepared as described by Nicolson & Blaustein (1972) and Nicolson *et al.* (1974). Crotin I and II were prepared as described by Stirpe *et al.*

(1976). Diphtheria toxin, purified by the method of Pope & Stevens (1958), was obtained from the Istituto Sieroterapico e Vaccinogeno Toscano 'Sclavo' (Siena, Italy); from this material, diphtheria toxin fragment A was prepared as described by Drazin *et al.* (1971). [$8\text{-}^3\text{H}$]Puromycin (3.7 Ci/mmol), [adenosine- ^{14}C]NAD $^{+}$ (260 mCi/mmol), U- ^{14}C -labelled protein hydrolysate (57 mCi/mg-atom of C), [$\gamma\text{-}^{32}\text{P}$]GTP (1019 mCi/mmol), [^3H]GMP- $P(\text{CH}_2)_3\text{P}^*$ (3.6 Ci/mmol) and L- ^{14}C phenylalanine (477 mCi/mmol) were from The Radiochemical Centre, Amersham, Bucks., U.K. GTP, GMP- $P(\text{CH}_2)_3\text{P}$, poly(U) (ammonium salt), aminoacyl-tRNA synthetase (14 units/mg of protein) and *Escherichia coli* K12 Mo tRNA were from Miles Laboratories, Kankakee, IL, U.S.A. Mixed charged tRNA containing L- ^{14}C -phenylalanyl-tRNA (0.167 μCi of [^{14}C]phenylalanine/mg of tRNA; 461 mCi/mmol of L-phenylalanine) was purchased from New England Nuclear Corp., Boston, MA, U.S.A. L- ^{14}C Phenylalanyl-tRNA was prepared by acylation of *E. coli* K12 Mo tRNA with L- ^{14}C phenylalanine in the presence of purified aminoacyl-tRNA synthetase, as described by Zasloff & Ochoa (1971). Yeast tRNA was from Calbiochem, San Diego, CA, U.S.A. ATP, Norit A (activated charcoal) and puromycin dihydrochloride were

* Abbreviations: GMP- $P(\text{CH}_2)_3\text{P}$, guanosine (β,γ -methylene)triphosphate; GTPase, guanosine triphosphatase; EF 1, elongation factor 1; EF 2, elongation factor 2; A site, aminoacyl-tRNA site; P site, peptidyl-tRNA site.

products of Sigma Chemical Co., St. Louis, MO, U.S.A. Creatine phosphate and creatine kinase were from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany.

Sucrose-washed rat liver ribosomes were prepared as described by Staehelin & Falvey (1971). From this material NH_4Cl -washed ribosomes and puromycin-treated ribosomes were prepared as described by Montanaro *et al.* (1973), and stored at -80°C . *Artemia salina* ribosomes, prepared as described by Zasloff & Ochoa (1971), were stored at -20°C after the addition of 1 vol. of glycerol. The concentration of ribosomes was calculated from the E_{260} , by making the following assumptions: $E_{1\text{cm}}^{1\%} = 145$; 1 mg of ribosomes = 250 pmol.

EF 1 and EF 2 were obtained from rat liver 'pH 5 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 (Bermek, 1972). Unresolved EF 1 and EF 2 were obtained from rat liver 'pH 5 supernatant' by precipitation at between 25 and 65% $(\text{NH}_4)_2\text{SO}_4$ saturation (Felicetti & Lipmann, 1968). A similar enzyme fraction was prepared from *A. salina* post-ribosomal supernatant; the enzymes from rat liver and *A. salina* were interchangeable. The protein content of the enzyme preparations 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 20 mM-Tris/HCl buffer, pH 7.5, 150 mM- NH_4Cl , 9 mM-magnesium acetate and 1 mM-dithiothreitol (medium A) for rat liver ribosomes and 80 mM-Tris/HCl buffer, pH 7.4, 120 mM-KCl, 7 mM-magnesium acetate and 2 mM-dithiothreitol (medium B) for *A. salina* ribosomes.

Poly(U)-directed incorporation of phenylalanine into polypeptides. With rat liver ribosomes the assay was performed as described by Montanaro *et al.* (1973). With *A. salina* ribosomes the incorporation reaction was carried out for 30 min at 24°C in 0.25 ml of medium B containing 2 mM-GTP, 42 pmol of [^{14}C]phenylalanyl-tRNA, 200 μg of poly(U), 0.5 mg of the unresolved elongation-factors preparation and 4 pmol of ribosomes.

Peptidyl- ^3H puromycin reaction. The reaction was carried out as previously described (Montanaro *et al.*, 1975), except that incubation was at 0°C to avoid the possibility of translocation of nascent peptidyl-tRNA from the A to the P site during the assay (Steinert *et al.*, 1974).

Deacylated tRNA release during translocation. Rat liver ribosomes in 0.2 ml of medium A were allowed to react for 10 min at 37°C with GTP and EF 2. At the end of incubation, the samples were filtered

through nitrocellulose membrane filters (Millipore HA; average pore size $0.45\mu\text{m}$), and the filters were washed twice with 0.2 ml of medium A. tRNA was measured in 0.3 ml of the combined filtrates and washings by aminoacylation with purified aminoacyl-tRNA synthetase. To each sample 2.5 μmol of ATP, 0.5 unit of aminoacyl-tRNA synthetase and 0.75 μCi of ^{14}C -labelled protein hydrolysate (2500 pmol of amino acids) were added in 0.2 ml of 220 mM-Tris/HCl buffer, pH 7.5, containing 25 mM-KCl, 11 mM- MgCl_2 and 8 mM-dithiothreitol. After 20 min of incubation at 37°C , the samples were cooled to 0°C , and 0.5 ml of 20% (w/v) cold trichloroacetic acid was added. The cold acid-insoluble radioactivity was collected on glass-fibre filters (Whatman GF/C), washed with 5% trichloroacetic acid and measured. The tRNA released is expressed as pmol of [^{14}C]aminoacyl-tRNA formed during the aminoacylation reaction.

Phenylalanyl-tRNA binding to ribosomes. With rat liver ribosomes the binding was performed as described by Sperti *et al.* (1975), except that puromycin was omitted from the assays.

With *A. salina* ribosomes the binding reaction was carried out in 0.25 ml of medium B containing 0.2 mM-GTP, 42 pmol of [^{14}C]phenylalanyl-tRNA, 200 μg of poly(U), 24 pmol of ribosomes and 180 μg of EF 1 protein. Since small amounts of contaminating EF 2 were present in the ribosomal preparation, the binding assay was preceded by inactivation of the enzyme by ADP-ribosylation: ribosomes were preincubated for 15 min at 24°C in 0.125 ml of medium B containing 1 mM- NAD^+ and 6 μg of diphtheria toxin fragment A. The volume was then brought to 0.25 ml by adding the components of the binding reaction, and incubation was continued for 15 min at 24°C .

EF 1- and EF 2-mediated binding of [^3H]GMP- $\text{P}(\text{CH}_2)_2\text{P}$ to rat liver ribosomes. The binding of [^3H]GMP- $\text{P}(\text{CH}_2)_2\text{P}$ mediated by EF 1 was assayed in reaction mixtures containing, in 0.25 ml, 60 mM-Tris/HCl buffer, pH 7.5, 80 mM- NH_4Cl , 6 mM- MgCl_2 , 2 mM-dithiothreitol, 12 μM - ^3H]GMP- $\text{P}(\text{CH}_2)_2\text{P}$ (diluted with unlabelled carrier to a specific radioactivity of 150 d.p.m./pmol), 100 μg of poly(U), 60 μg of mixed charged tRNA (containing 22 pmol of [^{14}C]phenylalanyl-tRNA), 106 pmol of NH_4Cl -washed ribosomes and 450 μg of EF 1 protein.

The binding of the nucleotide mediated by EF 2 was assayed in reaction mixtures containing, in 0.25 ml, 50 mM-Tris/HCl buffer, pH 7.5, 80 mM- NH_4Cl , 5 mM- MgCl_2 , 10 mM-dithiothreitol, 12 μM - ^3H]GMP- $\text{P}(\text{CH}_2)_2\text{P}$, 106 pmol of NH_4Cl -washed ribosomes and 108 pmol of EF 2.

After 15 min at 37°C , 0.23 ml of each sample was layered over 1.5 ml of 0.25 M-sucrose containing 60 mM-Tris/HCl buffer, pH 7.5, 80 mM- NH_4Cl , 6 mM- MgCl_2 and 2 mM-dithiothreitol, and centrifuged at 4°C in a Spinco Ti 50 rotor operated at 105 000 g_{av}.

for 150 min. Ribosomal pellets were rinsed twice with 8 ml of ice-cold water, and resuspended in 0.5 ml of water. A portion (0.1 ml) was used for the determination of ribosome concentration by measuring E_{260} , and 0.3 ml was counted for radioactivity.

GTPase assays. The EF 1- and EF 2-dependent GTPase activities of rat liver ribosomes were tested as previously described (Montanaro *et al.*, 1973; Sperti *et al.*, 1975). The EF 2-dependent GTPase activity of *A. salina* ribosomes was assayed in incubation mixtures containing, in 0.125 ml of medium B, 3 nmol of [γ - 32 P]GTP (diluted with unlabelled carrier to a specific radioactivity of 50 d.p.m./pmol); 27 pmol of ribosomes and 20 pmol of EF 2 were present, and incubation was at 24°C.

Reactivity of nascent peptides with puromycin during protein synthesis. Protein synthesis was carried out in reaction mixtures containing, in 5 ml of medium A, 1 mM-ATP, 0.4 mM-GTP, 10 mM-creatine phosphate, 250 μ g of creatine kinase, 2.5 mg of yeast tRNA, 3.0 units of aminoacyl-tRNA synthetase, 25 mg of unresolved elongation-factors preparation, 750 pmol of NH_4Cl -washed rat liver ribosomes and 25 μCi of ^{14}C -labelled protein hydrolysate (83330 pmol of amino acids). At time 0 and after 3 min of incubation at 37°C, four 0.1 ml samples were withdrawn. The remaining mixture was fractionated into 0.1 ml samples, and incubation was allowed to proceed at 37°C in the absence and in the presence of inhibitors (40 $\mu\text{g}/\text{ml}$). At selected times samples were withdrawn and cooled to 0°C. Incorporation of ^{14}C -labelled amino acids was measured at all times by adding to the samples 0.1 ml of 10% (w/v) trichloroacetic acid and counting the hot acid-insoluble radioactivity retained on glass-fibre filters. Samples withdrawn at time 0 and after 3 min and 10 min of total incubation at 37°C were supplemented with [^3H]puromycin

(diluted with unlabelled carrier to a specific radioactivity of 2200 d.p.m./pmol) and NH_4Cl to a final concentration of 10 μM and 320 mM respectively. After 20 min of incubation at 0°C, 0.1 ml of 10% (w/v) trichloroacetic acid was added, and the hot acid-insoluble radioactivity was collected and counted for ^{14}C and ^3H . No difference was observed at any time between the incorporation of ^{14}C assayed directly and that measured after incubation of ribosomes with puromycin at 0°C, indicating that at this temperature elongation factors were inactive.

Radioactivity measurements. Radioactivity was measured in a Packard Tri-Carb liquid-scintillation spectrometer. When a single isotope was present, filters and aqueous samples were counted after addition of 6 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]. The efficiency was approx. 65% for ^{14}C , 31% for ^3H and 60% for ^{32}P . For dual-labelling counting, methoxyethanol was omitted; the radioactive material was solubilized with 0.4 ml of 0.5 M-quaternary ammonium hydroxide in toluene (Soluene-350 from Packard Instrument Co., Downers Grove, IL, U.S.A.), and the samples were counted after addition of 10 ml of scintillation fluid. The efficiency for ^{14}C was approx. 50% on the ^{14}C channel and 12% on the ^3H channel; the efficiency for ^3H on the ^3H channel was approx. 44%.

Results

The two fractions of the extract of *Croton tiglium* (crotin I and II) inhibited poly(U)-directed incorporation of phenylalanine into peptides catalysed by both rat liver ribosomes (Figs. 1a and 1b) and *A. salina* ribosomes (Fig. 1c). When NH_4Cl -washed rat liver ribosomes were tested (Fig. 1a) a 100% inhibition

Fig. 1. Effect of crotin I, crotin II and ricin on poly(U)-directed incorporation of phenylalanine into polypeptides

(a) 50 pmol of NH_4Cl -washed rat liver ribosomes and 60 μg of mixed charged tRNA (22 pmol of [^{14}C]phenylalanyl-tRNA); (b) 50 pmol of puromycin-treated rat liver ribosomes and 60 μg of mixed charged tRNA (22 pmol of [^{14}C]phenylalanyl-tRNA); (c) 4 pmol of *A. salina* ribosomes and 42 pmol of [^{14}C]phenylalanyl-tRNA. ●, Control; ○, crotin I; △, crotin II; □, ricin.

was never reached, even with the largest doses of inhibitors. This effect is clearly related to the presence of endogenous peptide chains on ribosomes, since inhibition approached 100% when puromycin-treated rat liver ribosomes (Fig. 1b) and when *A. salina* ribosomes (Fig. 1c) were used; the former are stripped and the latter are naturally devoid (Hultin & Morris, 1968; S. Sperti, L. Montanaro, A. Mattioli, G. Testoni & F. Stirpe, unpublished work) of nascent peptides. In all cases the inhibition by croton II was much greater than that by croton I. With rat liver ribosomes the inhibition by croton II was of the same order as that previously reported for ricin (Montanaro *et al.*, 1973); with *A. salina* ribosomes the dose-effect curves of croton II and of ricin were practically coincident (Fig. 1c).

The results reported in Table 1 point to ribosomes as the component of the protein-synthesizing system that is sensitive to croton. In fact poly(U)-directed phenylalanine incorporation was strongly inhibited when rat liver ribosomes were preincubated with croton II (6 µg/ml) and washed twice by centrifugation. A similar inhibition was observed after preincubation of ribosomes with croton I (25 µg/ml), the residual activity being, in both cases, that observed when NH₄Cl-washed ribosomes were tested in the presence of added croton (Fig. 1a). In Table 1 (and the following Tables) the results for croton I are omitted, since no qualitative difference was ever observed between the effects of the two crotons on the partial reactions of peptide elongation.

The last column of Table 1 shows that the presence of croton II during preincubation of ribosomes did not affect the subsequent peptidyl-[³H]puromycin reaction. The increased reactivity with puromycin of ribosomes preincubated with EF 2 and GTP depends on both the integrity of the ribosomal peptidyl-transferase centre and translocation of peptide chains from the A to the P site during preincubation. The

normal reactivity with puromycin of croton-treated ribosomes indicates that the inhibitor did not affect the peptidyltransferase centre and allowed a complete EF 2-catalysed translocation. Translocation, however, might have occurred during preincubation before the action of the inhibitor on the ribosomes.

Table 2 shows the effect on translocation when ribosomes were preincubated with either croton II or ricin and then allowed to interact with EF 2 and GTP without removing the inhibitor. In Expt. 1, the increased reactivity with puromycin observed when GTP alone was added to ribosomes was apparently supported by catalytic amounts of EF 2 present in the sucrose-washed ribosomal preparation (approx. 4 pmol of EF 2/100 pmol of ribosomes; Schneider & Maxwell, 1973), since the effect could be removed by pretreating ribosomes with NAD⁺ and diphtheria toxin fragment A to inactivate the enzyme, or by washing the ribosomes free of elongation factors through sucrose gradients containing NH₄Cl

Table 1. *Effect of preincubation of rat liver ribosomes with croton II on the poly(U)-directed [¹⁴C]phenylalanine incorporation and on the peptidyl-[³H]puromycin reaction*

Sucrose-washed ribosomes (50 pmol/ml) were preincubated in the absence and in the presence of EF 2 and GTP; croton II (6 µg/ml) was present where indicated. Ribosomes were isolated from the preincubation mixtures, washed twice through sucrose gradients containing 0.6 M-NH₄Cl, and tested for both poly(U)-directed [¹⁴C]phenylalanine incorporation and the peptidyl-[³H]puromycin reaction.

Preincubation additions	[¹⁴ C]Phenylalanine incorporated (d.p.m.)	[³ H]Puromycin reacted (pmol/100 pmol of ribosomes)
None	6613	4.34
EF 2, GTP	7015	9.04
EF 2, GTP, croton II	1616	9.51

Table 2. *Effect of croton II and ricin on translocation*

In Expt. 1, 50 pmol of rat liver sucrose-washed ribosomes in 0.05 ml of medium A was preincubated for 10 min at 37°C in the absence and in the presence of 10 µg of croton II or ricin. For the translocation assay, samples were brought to 0.2 ml by the addition of medium A, and incubation was for 10 min at 37°C in the absence and in the presence of GTP (2 mM) and EF 2 (92 pmol). Finally NH₄Cl and [³H]puromycin (diluted with unlabelled carrier to a specific radioactivity of 900 d.p.m./pmol) were added to a final concentration of 320 mM and 10 µM respectively, and the amount of peptidyl-[³H]puromycin formed in 20 min at 0°C was measured. In Expt. 2, 95 pmol of NH₄Cl-washed ribosomes and 5 µg of croton II or ricin were present during preincubation; translocation was carried out in the presence of 0.2 mM-GTP and 135 pmol of EF 2; the amount of deacylated tRNA released was measured as described in the Experimental section.

Preincubation additions	Addition to translocation assay ...	Expt. 1 [³ H]Puromycin reacted (pmol/100 pmol of ribosomes)			Expt. 2 tRNA released (pmol/100 pmol of ribosomes)	
		None	GTP	GTP+EF 2	GTP	GTP+EF 2
None		6.26	12.25	13.31	0.46	5.19
Croton II		5.96	9.59	11.39	0.95	5.86
Ricin		5.63	9.69	11.39	0.48	5.25

(results not shown). With such catalytic amounts of EF 2, translocation was approx. 40% inhibited by preincubation of ribosomes with either crotin II or ricin. When EF 2 was added to the system in molar excess with respect to ribosomes, the inhibition of translocation was decreased to approx. 25%.

A second assay of translocation, independent of polypeptide chain elongation, consists of measuring the amount of deacylated tRNA that is released from the ribosomal P site on incubation with EF 2 and GTP (Inoue-Yokosawa *et al.*, 1974). The results of Expt. 2, Table 2, confirm by this method that one round of translocation in the presence of added EF 2 is scarcely affected by preincubation of ribosomes with either crotin or ricin.

Table 3 shows the effect of crotin II and of ricin on the EF 1- and GTP-dependent binding of phenylalanyl-tRNA to ribosomes. Although ribosomes were preincubated with the inhibitors, and these were present in the reaction mixtures at concentrations that gave maximal inhibition of polyphenylalanine synthesis (Fig. 1), the inhibition of binding never exceeded 30%. The fact that the binding reaction is scarcely affected by crotin and the peptidyltransferase reaction is not affected at all (Table 1) may explain the incomplete inhibition of polyphenylalanine synthesis observed with ribosomes carrying nascent peptide chains (Fig. 1a).

Both elongation factors bind to ribosomes in the presence of a guanine nucleotide, and catalyse a ribosome-dependent GTP hydrolysis in reactions that are uncoupled from, but related to, their function in polypeptide chain elongation. The experiments reported in Table 4 show the EF 1- and EF 2-mediated binding to ribosomes of the non-hydrolysable analogue of GTP GMP- $P(CH_2)P$ and the concomitant binding of phenylalanyl-tRNA that occurs in the presence of EF 1. The binding of GMP- $P(CH_2)P$ measures in an indirect way the binding of the two elongation factors to ribosomes (Richter, 1973). The

results clearly indicate that both crotin and ricin do not inhibit significantly the EF 1-dependent binding of the nucleotide and of aminoacyl-tRNA to ribosomes. On the contrary, a strong inhibition of the EF 2-mediated binding of the nucleotide was observed, indicating a decreased interaction of EF 2 with ribosomes. The data in Table 5 show that crotin II and ricin inhibited the EF 1-dependent and stimulated the EF 2-dependent GTPase activity of rat liver ribosomes. Stimulation is not in conflict with the decreased binding of EF 2 to ribosomes (Table 4), since the formation of an unstable EF 2-GDP-ribosome complex would increase the rate of GTP hydrolysis (Obrig *et al.*, 1973). Neither crotin

Table 3. Effect of crotin II and ricin on the enzymic binding of [^{14}C]phenylalanyl-tRNA to rat liver and *A. salina* ribosomes

NH₄Cl-washed rat liver ribosomes (70 pmol) were preincubated in the absence and in the presence of inhibitors (10 μ g) for 10 min at 37°C in 0.125 ml of 60 mM-Tris/HCl buffer, pH 7.5, containing 80 mM-NH₄Cl, 6 mM-MgCl₂ and 2 mM-dithiothreitol. *A. salina* ribosomes (24 pmol) were preincubated for 15 min at 24°C as described in the Experimental section; 10 μ g of crotin II or ricin were present where indicated. After preincubation all samples were brought to 0.25 ml for the aminoacyl-tRNA binding reaction; 120 μ g of mixed charged tRNA (containing 44 pmol of [^{14}C]phenylalanyl-tRNA) was present with rat liver ribosomes, and 42 pmol of [^{14}C]phenylalanyl-tRNA with *A. salina* ribosomes.

Preincubation additions	[^{14}C]Phenylalanyl-tRNA bound to	
	Rat liver ribosomes (d.p.m./100 pmol of ribosomes)	<i>A. salina</i> ribosomes (pmol/100 pmol of ribosomes)
None	5184	5.23
Crotin II	3627	4.53
Ricin	4470	4.86

Table 4. Effect of crotin II and ricin on the binding of GMP- $P(CH_2)P$ to ribosomes in the presence of EF 1 and EF 2

NH₄Cl-washed rat liver ribosomes (106 pmol) were preincubated in the absence and in the presence of inhibitors (10 μ g) for 10 min at 37°C in 0.1 ml of the appropriate buffer solution (see the Experimental section under 'EF 1- and EF 2-mediated binding of [3H]GMP- $P(CH_2)P$ to rat liver ribosomes'). After preincubation all samples were brought to 0.25 ml, and the binding of the nucleotide was assayed as described in the Experimental section. In Expt. 1, EF 1-mediated binding of [3H]GMP- $P(CH_2)P$ was carried out in the presence of [^{14}C]phenylalanyl-tRNA, and dual-labelling counting of radioactivity allowed the simultaneous determination of nucleotide and aminoacyl-tRNA bound.

Preincubation additions	Expt. 1				Expt. 2	
	[3H]GMP- $P(CH_2)P$ bound (pmol/100 pmol of ribosomes)		[^{14}C]Phenylalanyl-tRNA bound (d.p.m./100 pmol of ribosomes)		[3H]GMP- $P(CH_2)P$ bound (pmol/100 pmol of ribosomes)	
	EF 1 absent	EF 1 present	EF 1 absent	EF 1 present	EF 2 absent	EF 2 present
None	4.28	14.79	425	2472	3.97	64.93
Crotin II	1.19	13.91	387	2508	3.11	2.48
Ricin	2.52	12.16	333	2261	2.58	9.62

Table 5. Effect of crotin II and ricin on the EF 1-dependent and EF 2-dependent GTPase activity of rat liver ribosomes

In Expt. 1, the EF 1-dependent GTPase activity of ribosomes was tested in reaction mixtures containing, in 0.125 ml, 60 mM-Tris/HCl buffer, pH 7.5, 80 mM-NH₄Cl, 6 mM-MgCl₂, 2 mM-dithiothreitol, 30 µg of poly(U), 45 µg of mixed charged tRNA and 3 nmol of [γ -³²P]GTP; 60 pmol of NH₄Cl-washed ribosomes, 150 µg of EF 1 protein and 5 µg of crotin II or ricin were present where indicated. In Expt. 2, the EF 2-dependent GTPase activity of ribosomes was tested in reaction mixtures containing, in 0.125 ml, 50 mM-Tris/HCl buffer, pH 7.5, 80 mM-NH₄Cl, 10 mM-MgCl₂, 16 mM-dithiothreitol and 3 nmol of [γ -³²P]GTP; 63 pmol of NH₄Cl-washed ribosomes, 17 pmol of EF 2 and 5 µg of crotin II or ricin were present where indicated.

Expt.	Enzyme	Inhibitor	[γ - ³² P]GTP hydrolysed (pmol)			Enzyme-dependent GTPase activity of ribosomes	
			Ribosomes	Enzyme	Ribosomes + enzyme	(pmol of [γ - ³² P]GTP hydrolysed)	Activity (%)
1	EF 1	None	296	388	1177	493	100
		Crotin II	313	378	858	167	34
		Ricin	278	380	875	217	44
2	EF 2	None	154	115	1326	1057	100
		Crotin II	221	181	2070	1668	158
		Ricin	184	156	1819	1479	140

Fig. 2. Effect of crotin II and ricin on protein synthesis and on the reactivity of ribosomal nascent peptides with puromycin

The protein-synthesizing mixture is described in the Experimental section (see under 'Reactivity of nascent peptides with puromycin during protein synthesis'). After 3 min of incubation at 37°C, 0.1 ml samples, containing 15 pmol of NH₄Cl-washed rat liver ribosomes, were supplemented with inhibitors (4 µg); ●, control; △, crotin II; □, ricin. Samples were analysed for incorporation of ¹⁴C-labelled amino acids (—) and for reactivity of ribosomal nascent peptides with [³H]puromycin at 0°C (---), as described in the Experimental section. Each point is the mean of duplicate assays from two experiments. The S.E.M. was less than 2% for each point and is omitted for clarity.

nor ricin affected the EF 2-dependent GTPase activity of *A. salina* ribosomes (results not shown).

In an attempt to ascertain whether the EF 1-catalysed or the EF 2-catalysed step is primarily affected in the complete elongation cycle, an experiment was designed to localize the position of peptide chains on ribosomes when protein synthesis comes to a halt by the action of the inhibitors. NH₄Cl-washed ribosomes carry nascent peptides, about half of which are released by puromycin in the absence of translocation (Table 1), suggesting that they are more or less evenly distributed between the A and P sites. Nascent peptides of NH₄Cl-washed ribosomes were labelled by incubating ribosomes in the absence of poly(U) for 3 min at 37°C in a complete protein-synthesizing system, containing ¹⁴C-labelled amino acids. Inhibitors were then added, and, after further incubation for 7 min at 37°C, the reactivity of nascent peptides with [³H]puromycin was tested at 0°C. At this temperature only ribosomal peptidyl-tRNA bound to the P site reacts promptly with puromycin (Inoue-Yokosawa *et al.*, 1974); moreover, at this temperature protein synthesis and EF 2-catalysed translocation were completely blocked, also in the absence of inhibitors. The results are shown in Fig. 2. The reactivity of nascent peptides with puromycin increased during the first 3 min of incubation reaching a stable value, which was not modified by further incubation in the absence of inhibitors. Protein synthesis was 68% inhibited by crotin II and 63% by ricin 7 min after the addition of inhibitors. A parallel fall in the reactivity of nascent peptides with puromycin was observed, the value approaching that observed before ribosomes were engaged in protein synthesis. The results indicate that in our experimental conditions most of the peptide chains are located in the P site during protein

synthesis, and that some of these shift to the A site when synthesis comes to a halt by the action of crotin or ricin. This might indicate that inhibition of translocation prevails over inhibition of aminoacyl-tRNA binding.

Discussion

It has been suggested (Sperti *et al.*, 1975) that ricin inhibits protein synthesis in eukaryotic systems by affecting a 'GTPase site' localized on the larger ribosomal subunit, common to the two elongation factors. The hypothesis was based on the finding that, although ricin did not significantly inhibit the enzymic binding of aminoacyl-tRNA (Montanaro *et al.*, 1973; Sperti *et al.*, 1975), it prevented the formation of a stable complex between EF 2 and ribosomes (Montanaro *et al.*, 1975) and affected both EF 1- and EF 2-dependent GTPase activities of rat liver ribosomes (Sperti *et al.*, 1975). The data in the present paper indicate that crotin acts on the partial reactions of elongation in a way very similar to that of ricin.

Although from our results crotin and ricin scarcely affect the enzymic binding of aminoacyl-tRNA to both rat liver and *A. salina* ribosomes, Carrasco *et al.* (1975) report that ricin significantly inhibits the enzymic binding of aminoacyl-tRNA to rabbit reticulocyte ribosomes. The reason for the discrepancy between our and their results is presently unknown. It is also not clear why, in spite of the most rigorous control of experimental conditions, ricin in some experiments inhibited (Montanaro *et al.*, 1973; Sperti *et al.*, 1975) and in other ones enhanced (Table 5) the EF 2-dependent GTPase activity of rat liver ribosomes nor why ricin and crotin do not affect the EF 2-dependent GTPase activity of embryonic *A. salina* ribosomes. The finding that, after inactivation of a rat liver protein-synthesizing system by either ricin or crotin peptide chains are mainly confined to the A site, suggests that the main action of the inhibitors is not on the binding of aminoacyl-tRNA, but rather on translocation.

The damage of a common ribosomal site involved in the interaction with both elongation enzymes has also been proposed to explain the mechanism of action of an anti-viral peptide from *Phytolacca americana* (Obrig *et al.*, 1973). It is hard to conceive how all these substances may act by an identical mechanism, unless the ribosomal GTPase site is so exposed and so vulnerable that different chemical modifications lead to the same functional disorders. Alternatively, it can be suggested that all these plant proteins contain an active fragment, which induces the same chemical modification in ribosomes. Abrin (McPherson & Rich, 1973; Olsnes *et al.*, 1974b) and ricin (Pappenheimer *et al.*, 1974; Olsnes *et al.*, 1974a; Refsnes *et al.*, 1974) are known to consist of two fragments, one of which, the effectomer, is toxic

in vitro, and the other, the haptomer, is able to bind to the cell surface, allowing the penetration of the toxic fragment. Crotins, which have low toxicity *in vivo* (Stirpe *et al.*, 1976), may contain a fragment very similar to the effectomer of abrin and ricin, but differ in the remaining part of the molecule.

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