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Binding of Ricin A Chain to Rat Liver Ribosomes: Relationship to Ribosome Inactivation

Mary L. Hedblom, Daniel B. Cawley, Sophie Boguslawski, and L.L. Houston

Department of Biochemistry, University of Kansas, Lawrence, Kansas 66045

Ricin A chain was radioactively labeled using reductive alkylation, lactoperoxidase catalyzed iodination, and reaction with iodoacetamide or N-ethylmaleimide (NEM). The inhibition of cell-free rat liver protein synthesis by the modified A chains and the ribosome binding characteristics of each of the labeled derivatives was examined. [^{3}H]NEM was found to quantitatively react with the A chain sulfhydryl group normally involved in a disulfide bond with the B chain in intact ricin. Labeling the protein with [^{3}H]NEM had no effect on the in vitro inhibition of protein synthesis by the A chain. [^{3}H]NEM-labeled A chain binds to rat liver ribosomes in a manner which is dependent on the concentrations of NaCl and Mg^{2+}. At optimal Mg^{2+} concentration (5.5 mM), A chain binding to ribosomes is saturable and fully reversible either by dilution of the reaction mixture or by addition of unlabeled A chain. At 5.5 mM Mg^{2+}, A chain was found to bind to a single site on rat liver ribosomes with a dissociation constant of 6.2×10^{-8} M. [^{3}H]NEM-labeled A chain did not bind to isolated 40S ribosomal subunits and bound to 60S ribosomal subunits with a 1:1 molar stoichiometry and a dissociation constant of 2.2×10^{-7} M. The relationship between ribosome binding and A chain inhibition of eucaryotic protein synthesis is discussed.

Key words: ricin, lectin, toxin, ribosome, rat liver, wheat germ, N-ethylmaleimide

Ricin is a highly toxic glycoprotein found in the seeds of Ricinus communis, commonly known as castor beans. Reduction with 2-mercaptoethanol splits ricin into its two constituent polypeptide chains: The nontoxic B chain binds to galactose-containing receptors on the cell surface and thus facilitates the entry of the A chain or intact toxin

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into the cytoplasm [1, 2]; the A chain alone is capable of catalytically inactivating ribosomes in many cell-free eucaryotic systems [3] by interacting with the 60S ribosomal subunit. Intact ricin is needed for expression of in vivo toxicity, while the A chain may need to be liberated from the B chain in order to express in vitro inhibition of protein synthesis [3, 4]. Although the exact mechanism by which the ricin A chain damages the 60S ribosomal subunit is not yet known, the action probably causes inhibition of both the initiation and elongation phases of protein synthesis [5].

We have shown that ricin binds to a single site on eucaryotic ribosomes, presumably through its carbohydrate binding B chain, and that this binding is inhibited by lactose or galactose [6]. Since the B chain is not required for in vitro inhibition of protein synthesis and since ricin still inhibits protein synthesis in the presence of lactose [3], this binding is probably not involved in the inactivation of eucaryotic ribosomes. These data are not consistent with the hypothesis that a lectin-like molecule in the ribosome interacts with carbohydrate constituents covalently attached to ricin, because ricin has been reported to contain only mannose, glucose, glucosamine, and N-acetylglucosamine, and no galactose or galactose-based sugars have been detected [4, 7]. These data imply the existence of a galactose-containing structure in rat liver ribosomes.

Howard and Schnebli [8] have provided evidence for a carbohydrate-containing constituent in chicken liver ribosomes, and they have isolated a ribosomal glycoprotein using affinity chromatography on concanavalin A-Sepharose. Binding of concanavalin A to this constituent does not cause inhibition of polyuridylic acid translation by chicken liver ribosomes [8].

We have demonstrated that the binding of ^{3}H-reductively alkylated ricin A chain to rat liver ribosomes is specific for the 60S subunit and not the 40S subunit, consistent with the known site of action of ricin [6]. Furthermore, Escherichia coli ribosomes, which are resistant to the inhibitory action of ricin, do not bind ricin A chain. Our former studies were complicated by a component of the binding which was irreversible as measured by the inability of added unlabeled A chain to displace all of the bound labeled A chain from the ribosomes. The ribosome binding of ricin A chain appeared to be complex as reflected by curvature of the Scatchard plot. The plot extrapolated to two sites for the binding of ricin A chain to the 60S subunit and indicated the presence of both high-and low-affinity sites on the ribosomes. Since curved Scatchard plots are difficult to interpret [9], we synthesized additional derivatives of the A chain and analyzed their binding with the hope of finding a more suitable derivative. The results we report here indicate that the irreversible portion of the binding was a property induced by the reductive alkylation labeling procedure, and that low-affinity binding sites could be eliminated by using optimal magnesium concentration.

MATERIALS AND METHODS

A single species of ricin was isolated from castor beans and its A and B chains purified as previously described [6]. The A chain concentration was determined according to Murphy and Kies [10] and the B chain concentration by using the extinction coefficient reported by Olsnes et al [11]. Rat liver ribosomes were isolated and treated in separate steps with 0.5% sodium deoxycholate, 25 ng/ml pancreatic RNase (Sigma Chemical Co.)

for 10 minutes at 37°C, and 0.5 M KCl–0.2 mM puromycin as described before [12]. These treatments served to remove mRNA and membrane fragments and to strip initiation factors, elongation factors, and tRNA from the ribosomes. The concentration of rat liver ribosomes was determined assuming 15.33 pmole/A_{260} unit [13, 14]. Wheat germ ribosomes and rat liver ribosomal subunits were prepared as previously described [12] as were S-100 fractions from both sources. Protein synthesis was assayed with polyuridylic acid as message after the ricin A chain was neutralized with the 50% ammonium sulfate fraction of rabbit anti-ricin serum [12, 15]. The purity of the 40S and 60S ribosomal subunits was estimated by assaying polyuridylic acid translation using 0.05 A_{260} of 40S and 0.1 A_{260} unit of 60S subunits, alone or in combination. The 40S subunit preparation synthesized no detectable [^{3}H] polyphenylalanine while the 60S subunit preparation showed 9% of the level of polyphenylalanine synthesis obtained by mixing the 60S and 40S subunits.

Labeling Procedures

Ricin A chain was labeled using the procedures described below. In all cases, free reagent was removed by applying the sample to a Sephadex G-100 column equilibrated in 5 mM NaH_2PO_4 (pH 7.6) containing 200 mM NaCl or by dialyzing against the same buffer. Ricin A chain was iodinated using Na[^{125}I] (2.27 Ci/μmole, Amersham Corp.), lactoperoxidase and H_2O_2 essentially according to Marchalonis [16]. About 0.25 pmole ^{125}I was incorporated per pmole A chain. The A chain was reductively alkylated using [^{14}C]-formaldehyde (45.45 Ci/mole, New England Nuclear) and sodium borohydride according to Rice and Means [17]. In typical experiments, about 1.0 pmole [^{14}C] formaldehyde reacted per pmole A chain. Ricin A chain was reacted in the dark with a 20-fold molar excess of [^{14}C] iodoacetamide (15.76 Ci/mole, Amersham Corp) in 50 mM Tris-HCl (pH 8) for 1.5 hours at room temperature. Typically, 0.4–0.6 pmole [^{14}C] iodoacetamide reacted per pmole A chain.

N-[Ethyl-2-^{3}H] maleimide ([^{3}H] NEM, 150 Ci/mole, New England Nuclear) was supplied in pentane solution and most of the pentane was evaporated under a gentle stream of nitrogen immediately before the labeling reaction. The ricin A chain (3 mg in a final volume of 2 ml containing 20 mM NaH_2PO_4, pH 7) was reacted with a 20-fold molar excess of [^{3}H] NEM for 1.5–2 hours at room temperature. In typical experiments, between 0.84–1.0 pmole [^{3}H] NEM reacted per mole A chain to give about 100 cpm/pmole A chain. [^{3}H] NEM-labeled A chain was stored at –80°C and was stable for up to 6 months as measured by its ability to bind to ribosomes or inhibit poyuridylic acid translation.

Binding Assay and Analysis

The binding of ricin A chain to rat liver ribosomes was determined as follows: 18 pmoles of rat liver ribosomes were reacted with a 1–40-fold molar excess of labeled A chain in a final reaction volume of 200 μl containing 25 mM Tris-acetate (pH 7.5), 50 mM potassium acetate, 1.0 mM $CaCl_2$, and NaCl and magnesium acetate as indicated in the figure legends. At the end of 10 minutes incubation at 37°C, 175-μl samples were centrifuged for 30 minutes at 30 psig in a Beckman Airfuge (165,000g). The supernatant was carefully removed and if appropriate, three 50-μl aliquots were counted in 5 ml Brays solution. The counting efficiency was 42% for ^{3}H and 90% for ^{14}C. The tip of each nitrocellulose tube was cut off with a razor blade and placed in a 10 X 75 mm test tube. The ribosome pellet was solubilized in two successive 50-μl portions of 3% sodium dodecyl sulfate by

heating the tubes to 90°C and vigorously shaking on a Vortex mixer. The combined 100-μl sample was counted in 5 ml Brays solution with a counting efficiency of 41% for ^{3}H.

The binding of ricin A chain (A) to ribosomes (R) can be described as an equilibrium reaction

$$R + A \rightleftharpoons RA$$

where the dissociation constant (K_d) is defined as

$$K_d = \frac{[R]\,[A]}{[RA]}$$

Scatchard has derived an equation $\bar{\nu} = \eta[A]/(K_d + [A])$ which describes the binding of a ligand (A) to a macromolecule with multiple binding sites (η) which are noninteracting and which have the same dissociation constant [18]. In analyzing the binding of ricin A chain to ribosomes, $\bar{\nu}$ represents pmoles of labeled A chain bound per pmole ribosome, A is the molar concentration of free labeled A chain in the final reaction volume, and η is the number of binding sites per ribosome. Binding data were fitted to the Scatchard equation by varying both η and K_d using a weighted least-squares method and assuming equal variance for the $\bar{\nu}$ values [19]. All calculations were performed using a FORTRAN IV program [20] which provided values for K_d and η with the standard errors of their estimates.

RESULTS

We found previously that only 40% of the bound reductively alkylated ^{3}H-labeled ricin A chain could be removed from rat liver ribosomes when the complex was incubated with a large excess of unlabeled A chain [6]. This irreversible binding was incompatible with evidence from our laboratory and that of others [4] that the A chain acts enzymatically. Thus, we performed experiments to see if the native ricin A chain bound irreversibly or if this phenomenon was a consequence of the reductive alkylation procedure we used to label the protein. Rat liver ribosomes were incubated with buffer alone or buffer containing a 40-fold molar excess of unlabeled A chain. The two samples were then diluted 2-fold in buffer, and the ribosomes pelleted through a sucrose pad. The ribosomal pellets were resuspended and both samples subsequently exposed to a 10-fold excess of ^{3}H-reductively methylated A chain. The ribosomes which were pretreated with unlabeled A chain bound the same amount of labeled A chain as the control ribosomes. These data indicate that native ricin A chain binds reversibly to rat liver ribosomes and suggest that the irreversible binding we observed was an artifact introduced by the label or the conditions of the reductive alkylation procedure.

Activity, Reversibility, and Background Binding of Labeled A Chain Derivatives

In order to obtain a radioactively labeled molecule which was both fully catalytic and whose binding to ribosomes was completely reversible, we prepared several derivatives of ricin A chain by iodination and reaction with iodoacetamide and N-ethylmaleimide (Fig. 1). The conditions of iodination appear to be largely responsible for the 96% inactivation of the A chain since only 0.25 moles of ^{125}I were incorporated per mole of A chain. All of the

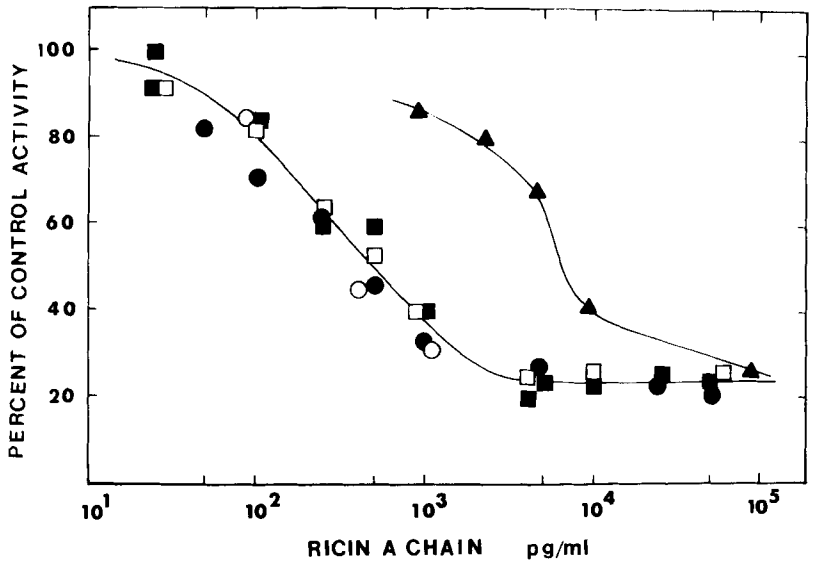

Fig. 1. Inhibition of polyuridylic acid translation in a rat liver cell-free system by ricin A chain. Ricin A chain and various labeled derivatives were assayed for their ability to inactivate rat liver ribosomes as described [12]. Briefly, ribosomes (2.5 pmoles) were treated with various concentrations of A chain for 10 minutes at 37°C. At this time, rabbit anti-ricin serum was added to stop any further ribosome inactivation. Protein-synthesis components were added and the reaction mixture incubated 45 minutes at 37°C. The symbols represent inhibition of ribosomes by control (■), [^{3}H] NEM-labeled (●), reductively alkylated (□), [^{14}C] iodoacetamide-labeled (○), and iodinated (▲) A chains.

other derivatives were fully active catalytically when compared to native A chain, indicating that the amino and sulfhydyl group(s) modified are not required for activity.

Our initial experiments showed that all of the A chain derivatives bound to rat liver ribosomes. To determine whether each labeled derivative bound reversibly, labeled A chain was reacted with ribosomes and then an excess of native A chain was added. The ribosome A chain complexes were pelleted by ultracentrifugation of the reaction mixture and the amount of labeled A chain still bound to the ribosome was compared with the amount predicted if the binding of the labeled A chain was freely reversible (assuming the appropriate dilution of the specific activity of the labeled A chain). Figure 2 demonstrates the competitive displacement of [^{3}H] NEM-labeled A chain from rat liver ribosomes by increasing amounts of unlabeled A chain. The line drawn through the experimental points represents the theoretical amount of displacement of the labeled A chain if a rapid equilibrium existed between free and bound A chain. By this criterion, the [^{3}H] NEM-labeled A chain binds reversibly to rat liver ribosomes. Table I shows that, as reported earlier [6], over half of the binding of reductively alkylated A chain is irreversible as measured by the competition assay. In contrast, all of the other derivatives bind reversibly to rat liver ribosomes.

In these binding studies, the net radioactivity bound to the ribosomal pellet was calculated by subtracting the background radioactivity (determined in the absence of ribosomes) from the gross cpm recovered from the nitrocellulose centrifuge tube. As shown in Table I, the background was very low for all of the labeled derivatives except for ^{125}I-labeled A chain. Using iodinated A chain, about 15% of the total radioactivity added

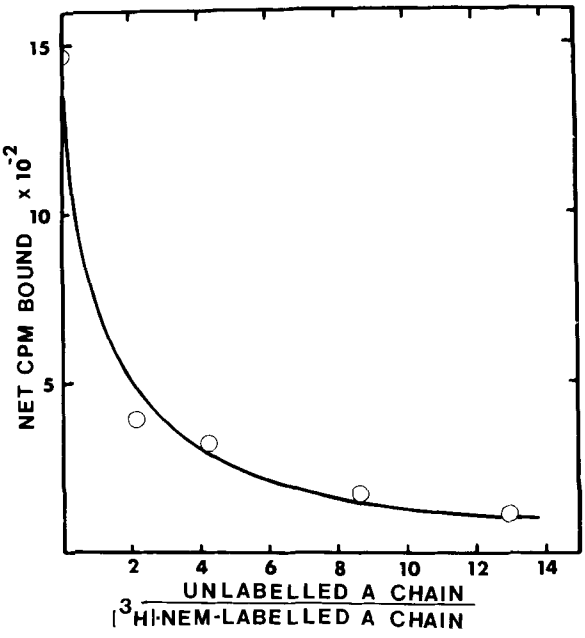

Fig. 2. Competition for binding sites using unlabeled A chain. Rat liver ribosomes (18 pmoles) were reacted with 156 pmoles [^{3}H] NEM-labeled A chain as described in Materials and Methods. At the end of a 5-minute incubation at 37°C, 342–2,052 pmoles of unlabeled A chain was added and the reaction mixture further incubated at 37°C for 5 minutes. Samples of the solution were centrifuged and net radioactivity recovered in the pellet plotted versus the molar excess of unlabeled A chain (○). The curve represents the radioactivity predicted by the dilution of the specific activity of labeled A chain.

TABLE I. Summary of the Binding Characteristics of Four Labeled A Chain Derivatives

Labeling reagent	Label incorporated in A chain (mole/mole)	Specific activity[a] in A chain (cpm/pmole)	Inhibition of rat liver protein synthesis compared to control A chain[b] (%)	Binding which is reversible[c] (%)	Background binding to nitrocellulose centrifuge tube[d] (%)
Na[^{125}I], H_2O_2, and lactoperoxidase	0.25	1,207[e]	6	100	15
[^{14}C] Formaldehyde and $NaBH_4$	1.0	64	100	40	3
[^{14}C] Iodoacetamide	0.2–0.6	8	100	100	1
[^{3}H] N-Ethylmaleimide	0.84–1.0	92	100	100	1

[a]The minimum specific activity obtained in typical labeling reactions is shown.
[b]Data are taken from Figure 1.
[c]Experiments were performed as described in Figure 2.
[d]The percent of total radioactivity added per tube which nonspecifically bound to the nitrocellulose tube in the absence of ribosomes is defined as the background binding.
[e]Na[^{125}I] was diluted 1:456 with KI before the reaction with A chain.

was nonspecifically bound to the nitrocellulose tube. In typical experiments, this background was large compared to the amount of radioactivity specifically bound to the ribosome pellet in control tubes. Because of this large nonspecific background and the fact that the iodination procedure inactivated the A chain, this derivative was unsuitable for use in further binding studies. [^{14}C] Iodoacetamide and [^{3}H] NEM-labeled A chain were found to be fully active and to bind reversibly to rat liver ribosomes with very low background binding. However, because of the low specific activity of the [^{14}C] iodoacetamide and because of its fractional incorporation into the A chain, we used [^{3}H] NEM-labeled A chain for all additional binding experiments reported here.

Inhibition of the Formation of the A-B Ricin Molecule by NEM

The extent of reaction of freshly isolated A chain with [^{3}H] NEM was determined as follows. The A chain was reacted with [^{3}H] NEM at 25°C, as described in Materials and Methods, in a final reaction volume of 100 μl. At various times, 5-μl aliquots were removed from the reaction mixture and added to 50 μl of 150 mM 2-mercaptoethanol containing 100 μg/ml bovine serum albumin as carrier. The samples were mixed and immediately precipitated by adding an equal volume of 10% trichloroacetic acid and were subsequently heated to 90°C for 2 minutes. The samples were filtered on glass fiber filters, washed with 5 ml of 5% trichloroacetic acid, and rinsed with 2 ml ice-cold ethanol; the filters were dried and the [^{3}H] NEM incorporated determined by scintillation counting. Saturation was achieved in 20 minutes with about 1 mole of NEM being incorporated per mole of protein, and increasing the reaction time up to 2 hours did not result in additional incorporation of label. The quantitative labeling of one thiol is consistent with the fact that reduction of ricin with 2-mercaptoethanol under mild conditions exposes a single sulfhydryl group in each of the toxin's subunits [7].

We considered it likely that NEM was reacting with the sulfhydryl group in the A chain which normally forms a disulfide bond with purified B chain and form [4, 7, 21]. To examine this possibility, we looked at the effect of [^{3}H] NEM labeling on the ability of the A chain to spontaneously re-form the disulfide bond with purified B chain and form intact ricin (Fig. 3). When A chain and B chain were incubated separately, only small amounts of A chain dimer and B chain dimer were formed (not detectable in the photograph). No detectable [^{3}H] NEM-labeled A chain dimers were formed following incubation of the isolated subunit. Ricin B and A chains quantitatively reoxidized to form ricin, in contrast to the reconstitution of only a small amount of 64,000 molecular weight ricin from the B chain and [^{3}H] NEM-labeled A chain. Since less than 1 mole of NEM was incorporated per mole of A chain in this particular NEM derivative, this small amount of ricin derivative probably results from the portion of A chain that was unlabeled during the reaction with [^{3}H] NEM. Considering these results and the specificity of NEM labeling, the site of reaction of NEM appears to be the free sulfhydryl group in the A chain.

Effect of NaCl on the Binding

Because our labeled A chain was stored in 5 mM NaH_2PO_4 (pH 7.6) containing 200 mM NaCl, we examined the effect of NaCl on the binding of [^{3}H] NEM-labeled A chain to rat liver ribosomes. In the presence of 5.5 mM Mg^{2+}, maximal binding was observed from 5 to about 30 mM NaCl. Further addition of NaCl drastically reduced the amount of binding while having a small effect on the recovery of the ribosomes by centrifugation. At 100 mM NaCl, the level of binding was about 45% of the level at 30 mM

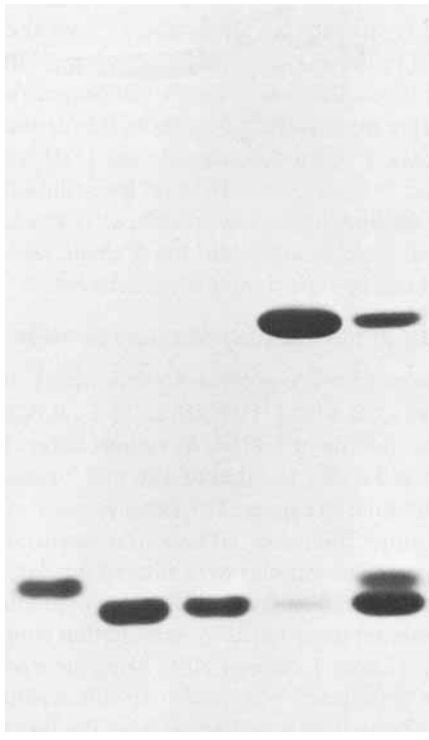

Fig. 3. Reoxidation of ricin subunits to form intact ricin. Protein samples were incubated for 1 hour at room temperature at a final concentration of 0.5 mg/ml in 100 mM NaCl, 10 mM 2-mercaptoethanol, 2.5 mM Na_2HPO_4 (pH 7.6). The samples were then dialyzed twice, for 4 hours each, against a large volume of the same buffer without 2-mercaptoethanol. The protein samples were then electrophoresed in 0.1% sodium dodecyl sulfate on a 12% polyacrylamide slab gel according to Laemmli [27] and the gel stained according to Fairbanks et al [28]. Protein samples contained, from left to right: 1) Ricin B chain; 2) ricin A chain; 3) [^{3}H]NEM-labeled ricin A chain (0.84 pmole [^{3}H]NEM/pmole A chain); 4) A chain plus B chain; 5) [^{3}H]NEM-labeled A chain plus B chain.

NaCl. The data were corrected by taking into account the amount of ribosomes which did not pellet. Similar results were obtained at 15 mM Mg^{2+}. We have found that the recovery of rat liver ribosomes by centrifugation varies with the particular preparation and depends on the concentrations of NaCl and Mg^{2+}, as discussed below. In all of the binding experiments reported here, conditions were chosen to obtain nearly quantitative pelleting (greater than 95%) of the ribosomes and optimal binding.

Mg^{2+} Dependency of the A Chain Inactivation of Wheat Germ and Rat Liver Ribosomes

We found that the extent of ribosome inactivation was influenced by the Mg^{2+} concentrations. Although Mg^{2+} exerts multiple effects upon ribosome structure and function, the influence of the metal ion on A chain inactivation could result from: 1) a direct protection of the ribosomes from inactivation by A chain; and/or 2) the partial restoration of residual activity of the damaged ribosomes.

In order to determine whether Mg^{2+} was exerting a direct protective effect against the A chain, we pretreated rat liver ribosomes with 0.2 ng/ml A chain at either low (8.8 mM) or high (17.3 mM) Mg^{2+}, added anti-ricin serum to neutralize the A chain, and then assayed protein synthesis in each case at high Mg^{2+} concentration (Table II). These data

indicate that Mg^{2+} has a small protective effect during the preincubation of ribosomes with A chain, perhaps because the ribosomes are less accessible to the toxin. The same level of inhibition was achieved when ribosomes were preincubated with A chain at either low or high Mg^{2+} and assayed in low Mg^{2+}. When A chain-treated ribosomes were assayed at low Mg^{2+}, any protective effect that Mg^{2+} may have had during the preincubation was masked. Better inhibition was achieved when Mg^{2+} was kept low.

We further investigated the abilities of different concentrations of Mg^{2+} to overcome the A chain-induced lesion using both rat liver and wheat germ ribosomes (Table III). Ribosomes were pretreated with ricin A chain for 10 minutes at 37°C, rabbit anti-ricin serum was added to stop further ribosome inactivation, and the residual polyuridylic acid translational activity assayed as described [12]. At 17 mM Mg^{2+} (Table III and [12]), wheat germ ribosomes were found to be unaffected by 2 μg/ml A chain, while decreasing the Mg^{2+} to 10mM resulted in 70% inhibition of polyuridylic acid translation by the same ribosomes. As shown in Table III, the residual activity of rat liver ribosomes was also increased at higher Mg^{2+} concentrations, although not as dramatically as for wheat germ ribosomes. However, there was a large difference in the amount of A chain needed to inhibit rat liver ribosomes compared to wheat germ ribosomes. Rat liver ribosomes were found to be about 5,000-fold more sensitive to ricin A chain than wheat germ ribosomes.

Effect of Mg^{2+} on A Chain Binding to Ribosomes

The results of protein synthesis experiments prompted us to examine the effect of Mg^{2+} on the binding of ricin A chain to rat liver ribosomes (Fig. 4). This experiment was

TABLE II. Ricin A Chain Inhibition of Polyphenylalanine Synthesis by Rat Liver Ribosomes

Magnesium concentration (mM)		Inhibition of rat liver protein synthesis (%)
Preincubation	Incubation	
8.8	17.3	26
17.3	17.3	10
8.8	8.8	56
17.3	8.8	54

Rat liver ribosomes were preincubated with 0.2 ng/ml A chain at low or high Mg^{2+} for 10 minutes at 37°C, the 50% ammonium sulfate fraction of rabbit anti-ricin serum was added to neutralize the A chain, and the ribosomes were subsequently assayed for polyphenylalanine synthesis at either low or high Mg^{2+}.

TABLE III. A Chain Inhibition of Polyphenylalanine Synthesis by Wheat Germ and Rat Liver Ribosomes as a Function of Mg^{2+}

Polyuridylic acid translation system	Concentration of ricin A chain in ng/ml	Percent inhibition observed at various magnesium concentrations		
		17 mM	13 mM	10 mM
Wheat germ	2000	0%	45%	70%
Rat liver	0.2	20%	34%	42%

Ribosomes were pretreated with ricin A chain and assayed as described in Table II. The same concentration of Mg^{2+} was present during both the preincubation and the subsequent protein synthesis reaction.

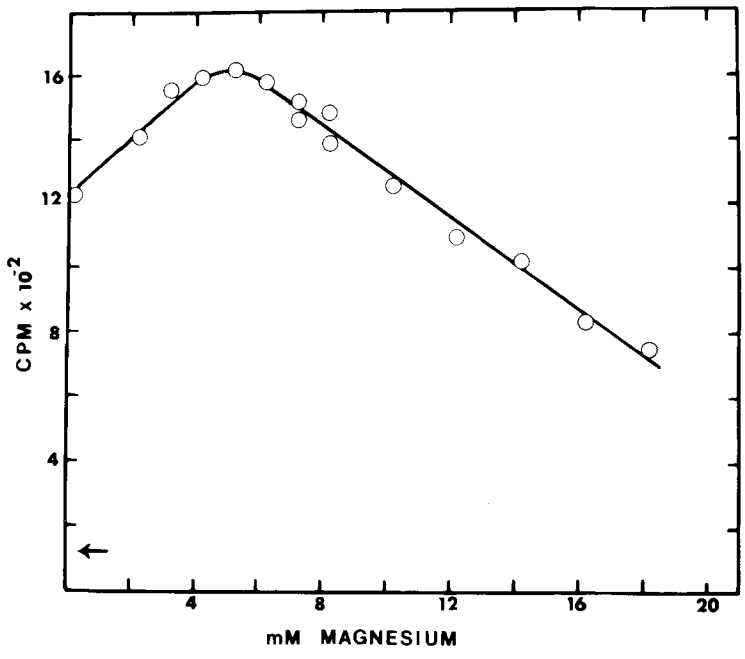

Fig 4. Effect of Mg^{2+} on the binding of A chain to rat liver ribosomes. Rat liver ribosomes (19 pmoles) were reacted with 139 pmoles [^{3}H]NEM-labeled A chain in the presence of 5 mM NaCl and varying amounts of magnesium acetate, as indicated. The gross cpm recovered in the pellet at the various Mg^{2+} concentrations are shown. The arrow shows the radioactive background in the absence of ribosomes.

performed in the presence of 1 mM $CaCl_2$ under the same ionic conditions used during polyuridylic acid translation. At Mg^{2+} concentrations below about 5 mM, a considerable portion of the ribosomes did not pellet. Taking into account the amount of ribosomes which did not pellet, we calculate that binding of ricin A chain to ribosomes is about the same from 2 to 6 mM Mg^{2+}. At Mg^{2+} concentrations below 2 mM or above 7 mM, the binding appeared to be much reduced. The reduced binding above 7 mM Mg^{2+} was not due to any change in the quantitative recovery of ribosomes after centrifugation.

Relationship of A Chain Binding to Ribosome Inactivation

From data such as presented in Figure 4, the fractional binding at various Mg^{2+} concentrations (compared to the maximal binding observed at 5.5 mM) can be calculated. The amount of protein synthesis inhibition observed following pretreatment of rat liver ribosomes with 0.2 ng/ml A chain at various Mg^{2+} concentrations can be obtained from experiments such as those reported in Table III. A large number of binding and protein synthesis experiments were performed, and the amount of binding observed at a particular Mg^{2+} concentration was plotted versus the protein synthesis inhibition observed at the same Mg^{2+} concentration. The decrease in binding of ricin A chain with increasing Mg^{2+} is linearly related to the corresponding increase in residual activity of toxin-treated ribosomes as shown in Figure 5.

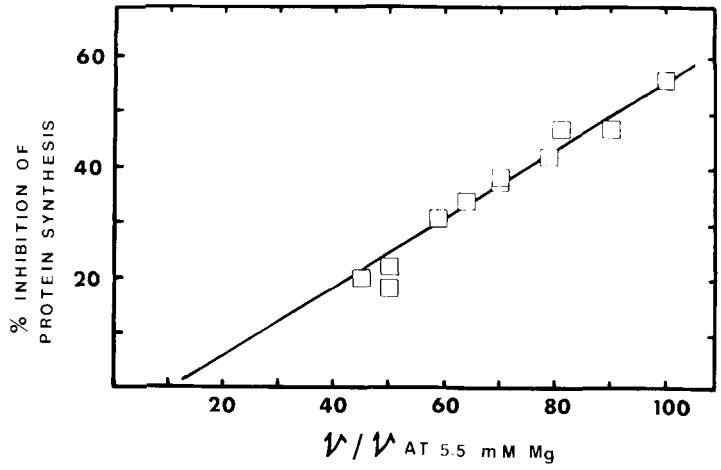

Fig. 5. Correlation between the decrease in binding of A chain to ribosomes and the decrease in protein synthesis inhibition by the A chain in the presence of increasing amounts of Mg^{2+}. The amount of rat liver ribosome A chain binding observed at various Mg^{2+} concentrations was expressed as a percent of the binding observed at 5.5 mM Mg^{2+} (see text). This percent of A chain binding was then plotted versus the inhibition of rat liver polyuridylic acid translation by 0.2 ng/ml A chain observed at that same Mg^{2+} concentration. The latter was expressed as a percentage of the control synthesis without A chain treatment. Protein synthesis data were gathered by performing experiments as described in Figure 1 and Table II.

Binding Parameters of A Chain to Rat Liver Ribosomes and Ribosomal Subunits

The characteristics of the binding of ricin A chain to rat liver ribosomes in the presence of an optimal concentration of Mg^{2+} (5.5 mM) were then examined. In Figure 6, the pmoles of [^{3}H]NEM-labeled A chain bound per pmole rat liver ribosome ($\bar{\nu}$) is plotted versus the concentration of unbound A chain (A). At 5.5 mM Mg^{2+}, the data fit a rectangular hyperbola with saturation being achieved when about 15 moles of A chain were added per mole of ribosome. The data were fit to the Scatchard equation [18] using a weighted least-squares method [19, 20]. The solid line in Figure 6 is the graphical representation of the Scatchard equation when $\eta = 0.99 \pm 0.03$ and $K_d = 6.2 \times 10^{-8} \pm 0.6 \times 10^{-8}$M. Thus, at 5.5 mM Mg^{2+}, ricin A chain binds reversibly to a single site on rat liver ribosomes with a dissociation constant of 6.2×10^{-8}M.

In contrast, binding at higher (15.4 mM) Mg^{2+} concentrations did not saturate, even when over 70 moles of A chain were added per mole of ribosome. In Figure 7, binding data obtained at 15.4 mM Mg^{2+} are plotted according to the method of Scatchard [18]. The Scatchard plot was markedly curved, indicating the presence of multiple classes of binding sites and the presence of a large number of low-affinity binding sites. Klotz and Hunston [9] have discussed the difficulties involved in interpreting such complex binding.

Because the ricin-sensitive site is known to be on the 60S ribosomal subunit of rat liver ribosomes [22], we examined the binding of [^{3}H]NEM-labeled A chain to isolated 40S and 60S ribosomal subunits. No binding to 40S subunits was observed above background

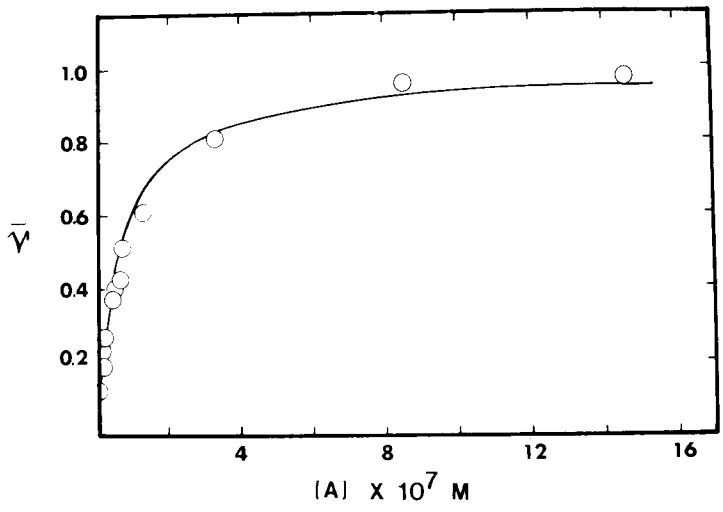

Fig. 6. Binding of [^{3}H]NEM-labeled A chain to ribosomes. Rat liver ribosomes (18 pmoles) were reacted with increasing amounts of A chain (up to 418 pmoles) in the presence of 5.5 mM Mg^{2+} and 20 mM NaCl and the pmoles of [^{3}H]NEM-labeled A chain bound per pmole of ribosomes ($\bar{\nu}$) plotted versus the concentration of unbound A chain (A) (○). These data were fitted to the Scatchard equation [18] $\bar{\nu} = \eta[A]/(K_d + [A])$ using a weighted least-squares method and assuming equal variance for the $\bar{\nu}$ values [19]. All calculation were performed using a FORTRAN IV program [20], which provided values for η (the number of A chain binding sites on the ribosome) and K_d (the dissociation constant as derived in Materials and Methods) and the standard errors of estimates. The solid line represents the best-fit hyperbola assuming $\eta = 0.99 \pm 0.03$ and $K_d = 6.2 \times 10^{-8} \pm 0.6 \times 10^{-8}$ M.

(data not shown). In contrast, labeled A chain bound to a single, high-affinity site on rat liver 60S ribosomal subunits (Fig 8). A weighted least-squares fit of the data indicated that $\eta = 1.02 \pm 0.05$ and $K_d = 2.2 \times 10^{-7} \pm 0.3 \times 10^{-7}$ M.

DISCUSSION

When ricin A chain was reductively alkylated using formaldehyde and sodium boro[^{3}H]hydride as described previously [6] or using [^{14}C]formaldehyde and $NaBH_4$ as described here, over half of its binding to rat liver ribosomes was irreversible. We have performed this reductive methylation reaction using many different batches of formaldehyde and $NaBH_4$ and always obtained a derivative which contained about one methyl group per mole of protein. In all cases, even though a proportion of the binding of the methylated A chain was irreversible (about 50–60%), the derivatized A chain was fully active when compared to underivatized A chain.

However, we have shown that the reaction of ricin A chain with ribosomes is freely reversible by demonstrating that ribosomes pretreated with unlabeled A chain (and washed through sucrose by centrifugation) subsequently reacted with labeled A chain as well as control ribosomes. Therefore, the initial binding of the underivatized A chain had no effect on the binding characteristics of the ribosomes. We have previously shown that both reduced ricin [6, 12] and ricin A chain are removed from the ribosome by centrifugation through a buffered sucrose pad. The hypothesis of an equilibrium reaction of ribosomes and ricin [6] or ricin A chain would also explain the observation of Lugnier [23] that

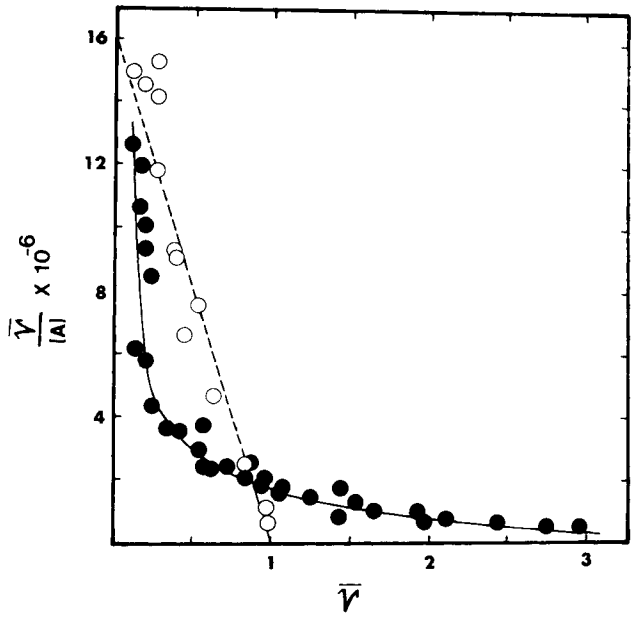

Fig. 7. Scatchard plots of the binding of [^{3}H]NEM-labeled A chain to ribosomes. Rat liver ribosomes (18 pmoles) were reacted with 18–1,300 pmoles of A chain in the presence of 30 mM NaCl containing 15.4 mM Mg^{2+} (•) or 5.5 mM Mg^{2+} (○) and the data plotted according to the binding equation of Scatchard [18], $\bar{\upsilon}/A = \eta K_a - \bar{\upsilon} K_a$, where $\bar{\upsilon}$ is pmoles of labeled A chain bound per pmole of ribosome, A is the molar concentration of free labeled A chain, η is the number of binding sites per ribosome and K_a is the association constant. The graphical representation of the data obtained at 5.5 mM Mg^{2+} (see Fig. 6) is shown in the dotted line.

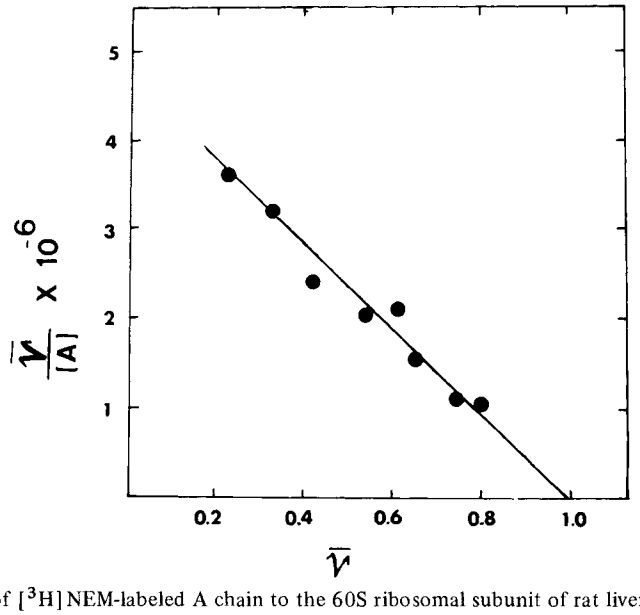

Fig. 8. Binding of [^{3}H]NEM-labeled A chain to the 60S ribosomal subunit of rat liver ribosomes. 60S ribosomal subunits (17 pmoles) were reacted with 28–278 pmoles of A chain at 5.5 mM Mg^{2+} and 10 mM NaCl and the data plotted according to Scatchard [18]. These data were analyzed as described in Figure 6 and the solid line represents the best-fit line assuming $\eta = 1.02 \pm 0.05$ and $K_d = 2.2 \times 10^{-7} \pm 0.3 \times 10^{-7}$M.

[^{125}I]-labeled ricin does not bind to rat liver ribosomes since he centrifuged the complex through a sucrose gradient. Such an assay would detect only irreversibly bound ricin.

In our hands, lactoperoxidase-catalyzed iodination of less than one tyrosine residue per mole of isolated A chain resulted in an 18-fold decrease in its ability to inhibit protein synthesis. This suggests that some inactivation of the A chain, not related to the number of iodine atoms incorporated, occurred during the reaction. This inactivation may have been due to oxidation of the A chain by H_2O_2, a possibility which may complicate the interpretation of other studies in which intact ricin was iodinated [23–26]. [^{125}I]-Labeled A chain bound to ribosomes in a reversible manner, but quantitation of the binding was precluded by the high level of nonspecific binding of this derivative to the nitrocellulose centrifuge tubes.

[^{3}H]NEM-Labeled A chain, which was fully active, fulfilled the criteria needed for binding assays (reversibility and low background binding to nitrocellulose centrifuge tubes) and it could be prepared with a high enough specific activity to obtain binding data at low input ratios of A chain to ribosomes. Funatsu et al [21] have determined the amino acid sequence of the carboxy terminal portion of the ricin A chain and their results indicated that the cysteine at the ninth position from the carboxy terminus participates in a disulfide bond to the B chain of ricin. Under the conditions used, we found that only one mole of [^{3}H]NEM was incorporated per mole of A chain and since the [^{3}H]NEM treatment prevented the A-B ricin molecule from forming, it is probable that the [^{3}H]NEM-labeled A chain used in these studies was labeled specifically at the ninth position from the carboxy terminus.

The association of the ricin A chain with the B chain through an interchain disulfide bond appears to place constraints on the ability of the A chain to act enzymatically on ribosomes [3, 4]. However, NEM labeling of the sulfhydryl freed from the interchain disulfide by reduction does not reduce the activity of the A chain [7 and this work] and indicates that the free sulfhydryl is not needed in order for the A chain to inhibit protein synthesis. We have shown before that the A chain can only bind to the ribosome when it is freed of the constraints imposed by the B chain [6]. This effect correlates well with the fact that the A chain is capable of inactivating the 60S ribosomal subunit only after it is released from the B chain by reduction. Immunological data [15] suggest that the inability of the A chain to bind to and inactivate ribosomes when bound to the B chain by a disulfide bond may be because the A chain is in a different conformation in intact ricin.

Ribosomes often cannot be completely inactivated by ricin or by abrin, a plant toxin which has a mechanism of action similar to that of ricin [4]. Our results indicate that ricin A chain is able to inhibit ribosomes better when Mg^{2+} is low and that the toxin-induced damage can be partly overcome by increasing the Mg^{2+} concentration. Olsnes et al [3] and Skorve et al [5] have made similar observations using ricin and abrin, respectively. We find that the residual activity of A chain-treated wheat germ ribosomes is acutely sensitive to Mg^{2+} concentration, while the effect of Mg^{2+} on A chain-treated rat liver ribosomes is less pronounced. In contrast to the results of Skorve et al [5] using abrin A chain, we found that Mg^{2+} can also afford some protection of the ribosomes during the preincubation of ribosomes with ricin A chain.

An optimal concentration of Mg^{2+} exists for the binding of the A chain to ribosomes, and there is a direct correlation between the decrease in binding of A chain to ribosomes at higher concentrations of Mg^{2+} and the corresponding increase in residual activity of A chain-treated ribosomes in polyuridylic acid translation. Therefore, it would appear that Mg^{2+} concentrations which allow optimal binding of ricin A chain to rat liver ribosomes

promote maximal ribosome inactivation. It should be emphasized, however, that this protective effect of Mg^{2+} is small compared to its ability to overcome A chain-induced ribosome damage.

One explanation for the protective effect is that Mg^{2+} may be affecting the structure of the ribosome and modifying the accessibility of the A chain binding site as suggested by the more complex binding behavior observed at high Mg^{2+} (Fig. 7). At the optimal Mg^{2+} concentration, saturation is obtained with one mole of [^{3}H]NEM-labeled A chain bound per mole of ribosomes, and only one class of site is observed with a dissociation constant of 6.2×10^{-8}M. The Scatchard plot at high Mg^{2+} indicates a qualitative decrease in the number of high-affinity binding sites and the appearance of low-affinity binding sites not observed at optimal Mg^{2+}. The exact nature of the additional binding sites observed using less favorable conditions remains obscure. The decrease in high-affinity binding to ribosomes with increasing Mg^{2+} may be related to the protection against A chain inactivation observed at high Mg^{2+}.

The Scatchard plots of [^{3}H]NEM-labeled A chain binding to both 80S ribosomes and 60S ribosomal subunits are linear at optimal Mg^{2+}, in contrast to the curved plots observed at higher Mg^{2+} concentrations. The evidence presented here indicates that the binding of A chain to 80S ribosomes is about 3.5 times as strong as binding to the isolated 60S subunit, although the number of sites (one) remains the same. This difference in binding affinities may reflect some loss of integrity of the 60S subunit during the isolation procedure, or it may indicate that when the 60S subunit is complexed with the 40S subunit, it is in a conformation more susceptible to A chain binding.

Because of the binding specificity of the [^{3}H]NEM-labeled A chain and its enzymatic characteristics, we believed that the data obtained using this derivative reflect the action of native A chain on eucaryotic ribosomes and that the binding represents one of the steps necessary for ribosome inactivation by ricin. It is interesting to note that the K_d we have determined for the binding (0.6 to 2.2×10^{-7}M) is similar to the K_m for the inactivation of ribosomes by ricin A chain (2.0×10^{-7}M), as determined by Olsnes et al [29].

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REFERENCES

1. Refsnes K, Munthe-Kaas AC: J Exp Med 143:1464, 1976.
2. Sandvig K, Olsnes S, Pihl A: Eur J Biochem 82:13, 1978.
3. Olsnes S, Refsnes K, Pihl A: Nature (London) 249:627, 1974.
4. Olsnes S, Pihl A: In Cuatrecasas P (ed): "The Specificity and Action of Animal, Bacterial and Plant Toxins." New York: John Wiley and Sons, 1977, pp 130–173.
5. Skorve J, Abraham AK, Olsnes S, Pihl A: Eur J Biochem 79:559, 1977.
6. Hedblom ML, Cawley DB, Houston LL: Arch Biochem Biophys 177:46, 1976.
7. Olsnes S, Refsnes K, Christensen TB, Pihl A: Biochim Biophys Acta 405:1, 1975.
8. Howard GA, Schnebli HP: Proc Natl Acad Sci 74:818, 1977.
9. Klotz IM, Hunston DL: Biochemistry 10:3065, 1971.

10. Murphy JB, Kies MW: Biochim Biophys Acta 45:382, 1960.
11. Olsnes S, Refsnes K, Christensen TB, Pihl A: Biochim Biophys Acta 405:1, 1975.
12. Cawley DB, Hedblom ML, Hoffman EJ, Houston LL: Arch Biochem Biophys 182:690, 1977.
13. Baliga BS, Munro HN: Biochim Biophys Acta 277:368, 1972.
14. Hamilton MG, Pavlovec A, Petermann ML: Biochemistry 10:3424, 1971.
15. Olsnes S, Saltvedt E: J Immunol 114:1743, 1975.
16. Marchalonis JJ: Biochem J 113:299, 1969.
17. Rice RH, Means GE: J Biol Chem 246:831, 1971.
18. Scatchard G: Ann NY Acad Sci 51:660, 1949.
19. Wilkinson GN: J Biochem Tokyo 80:324, 1961.
20. Cleland WW: Adv Enzymol 29:1, 1967.
21. Funatsu G, Yoshitake S, Funatsu M: Agric Biol Chem 41:1225, 1977.
22. Sperti S, Montanaro L, Mattioli A, Stirpe F: Biochem J 136:813, 1973.
23. Lugnier AAJ: FEBS Lett 67:80, 1976.
24. Sandvig K, Olsnes S, Pihl A: J Biol Chem 251:3977, 1976.
25. Funatsu M, Funatsu G, Ishiguro M, Nanno S: Jpn J Med Sci Biol 23:264, 1970.
26. Funatsu G, Migauchi S, Yoshizuka N, Funatsu M: Agric Biol Chem 41:1217, 1977.
27. Laemmli UK: Nature (London) 227:680, 1970.
28. Fairbanks G, Steck TL, Wallach DFH: Biochemistry 10:2606, 1971.
29. Olsnes S, Fernandez-Puentes C, Carrasco L, Vazquez D: Eur J Biochem 60:281, 1975.