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High-pressure-liquid-chromatographic and fluorimetric methods for the determination of adenine released from ribosomes by ricin and gelonin

Mariacristina ZAMBONI, Maurizio BRIGOTTI, Fioretta RAMBELLI, Lucio MONTANARO* and Simonetta SPERTI

Dipartimento di Patologia Sperimentale dell'Università di Bologna, Via S. Giacomo 14, 40126 Bologna, Italy

The high fluorescence of adenine-containing compounds after reaction with chloroacetaldehyde was used to measure the adenine released from rat liver and *Artemia salina* ribosomes by the action of ricin A chain and gelonin, two ribosome-inactivating proteins (RIPs) that share the same mechanism of action, consisting in the hydrolysis of the *N*-glycosidic bond of A-4324 of 28 S rRNA. Two methods were employed: (i) h.p.l.c. of the chloroacetaldehyde-reactive material released by RIPs; h.p.l.c. associated with a fluorescence detector allows the identification of adenine and its dosage at quantities as low as 2 ng; (ii) the direct fluorimetric measurement of the material that had reacted with chloroacetaldehyde. The amount of adenine released increases when ribosomes are pretreated in conditions that lead to their dissociation into subunits. Adenine protects ribosomes from the inhibition by ricin A-chain. When ribosomes were incubated with ricin A-chain in the presence of [^{14}C]adenine no incorporation of radioisotope in ribosomes was observed, indicating that neither exchange nor reversal reactions occurred. A binding of [^{14}C]adenine to ricin A chain was not detected by equilibrium dialysis.

INTRODUCTION

Ricin and gelonin belong to a group of proteins widely distributed in the plant kingdom that inhibit protein synthesis by inactivating eukaryotic ribosomes. Ricin is a two-chain protein; the B-chain binds to cell receptors and the A-chain inactivates protein synthesis. Gelonin is a single-chain protein that behaves similarly to the A-chain of ricin. The observation that the target of ricin is the 60 S ribosomal subunit was made several years ago (Sperti *et al.*, 1973), but, in spite of a great deal of effort, the enzymic mechanism of action of ricin and related ribosome-inactivating proteins (RIPs) has only recently been elucidated. Endo *et al.* (1987) showed that the treatment of rat liver ribosomes with ricin A-chain makes the 5′- and 3′-phosphodiester bonds in position A-4324 of 28 S rRNA very susceptible to aniline hydrolysis, and this susceptibility is due to removal of the adenine base by specific hydrolysis of its *N*-glycosidic bond (Endo & Tsurugi, 1987). This enzymic activity is shared by all other RIPs of both plant and bacterial origin hitherto tested (Endo *et al.*, 1988*a*,*b*,*c*; Stirpe *et al.*, 1988).

The method commonly used to test the *N*-glycosidase activity of RIPs requires extraction of RNA from the inactivated ribosomes, treatment of RNA with aniline and individuation of the 'aniline fragment' by gel electrophoresis. The presence of contaminant RNAases in the RIP preparations or a contamination with nucleases during the handling of RNA often leads to complicated electrophoretic patterns. Before the observation by Endo *et al.* (1987), RNAase contamination has been a major problem in the investigation of the mechanism of action of RIPs, and a nuclease activity on 5 S and 5.8 S rRNA has been proposed as relevant to the mechanism of ribosome inactivation by some RIPs (Obrig *et al.*, 1985).

The present paper describes a rapid method for the quantitative determination of the adenine removed by RIPs. The method is based on the conversion of adenine into its 1,N^6-etheno derivative by chloroacetaldehyde (Barrio *et al.*, 1972). The use of h.p.l.c. with a fluorescent detector allows the identification of adenine.

EXPERIMENTAL

Materials

Ricin, adenosine, 3′-AMP, 5′-AMP and poly(U) were from Boehringer Mannheim (Mannheim, West Germany). Gelonin (M_r 30500) was generously given by Professor F. Stirpe (of our Department). The A-chain of ricin (M_r 30000) was prepared by the method of Sperti *et al.* (1986). Adenine was from Nutritional Biochemicals Corp. (Cleveland, OH, U.S.A.). tRNA^{Phe} was from Sigma Chemical Co. (St. Louis, MO, U.S.A.). [^{14}C]Adenine (57 mCi/mmol), L-[^{14}C]leucine (348 mCi/mmol) and L-[^{14}C]phenylalanine (513 mCi/mmol) were from The Radiochemical Centre (Amersham, Bucks., U.K.). tRNA^{Phe} was charged with L-[^{14}C]phenylalanine by the method of Hultin & Näslund (1978). Ethanol was Rudi Pont from Eurobase (Milano, Italy), and RNAase-free sucrose was from Bio-Rad Laboratories (Richmond, CA, U.S.A.). Chloroacetaldehyde was prepared from its dimethyl acetal (Merck–Schuchardt, Darmstadt, West Germany) by the method of McCann *et al.* (1983). Unfractionated rabbit reticulocyte lysate was prepared as described by Allen & Schweet (1962). Ribosomes and post-ribosomal supernatant enzyme fractions were pre-

Abbreviation used: RIP, ribosome-inactivating protein.

* To whom correspondence should be addressed.

pared from rat liver by the method of Stahelin & Falvey (1971) and from *Artemia salina* undeveloped embryos by the method of Sierra *et al.* (1974).

Methods

Unless otherwise stated the saline compositions of the buffers were: buffer A, 50 mM-triethanolamine/HCl buffer, pH 7.5, containing 1.5 mM-$MgCl_2$ and 500 mM-KCl; buffer B, 50 mM-Tris/HCl buffer, pH 7.8, containing 700 mM-KCl, 11 mM-magnesium acetate and 20 mM-2-mercaptoethanol; buffer C, 20 mM-Tris/HCl buffer, pH 7.5, containing 100 mM-NH_4Cl, 7 mM-magnesium acetate and 1 mM-dithiothreitol; buffer D, 80 mM-Tris/HCl buffer, pH 7.4, containing 120 mM-KCl, 7 mM-magnesium acetate and 2 mM-dithothreitol.

Ribosomes and protein synthesis. Protein synthesis with the rabbit reticulocyte lysate was performed in the standard system of Sargiacomo *et al.* (1983). Dissociation of ribosomes into subunits was obtained by KCl/puromycin treatment of rat liver ribosomes in buffer A (Blobel & Sabatini, 1971) and by KCl treatment of *A. salina* ribosomes in buffer B (Zasloff & Ochoa, 1971). The protein-synthesizing system with rat liver ribosomes was in 100 μl of buffer C containing 0.1 μmol of ATP, 0.06 μmol of GTP, 1 μmol of phosphocreatine, 30 μg of creatine kinase, 100 μg of post-ribosomal enzyme protein, 80 μg of poly(U), 300 pmol of [^{14}C]phenylalanine and, unless otherwise stated, 10 pmol of ribosomes; incubation was for 10 min at 37 °C. With *A. salina* ribosomes the protein-synthesizing system was in 100 μl of buffer D containing 2 μmol of GTP, 22 pmol of [^{14}C]phenylalanyl-tRNA, 80 μg of poly(U), 40 μg of 'S-105 supernatant' and 2.5 pmol of ribosomes; incubation was for 20 min at 24 °C. After incubations, hot-acid-insoluble radioactivity was collected on glass-microfibre filters (Whatman GF/C) as described by Montanaro *et al.* (1978) and, after addition of 5 ml of Ready-Gel (Beckman Instruments, Fullerton, CA, U.S.A.), measured in an LKB liquid-scintillation counter.

Fluorescence assay of the adenine released by RIPs. Experiments were performed by incubating ribosomes (3–5 nmol/ml) in the appropriate buffer (buffer C, for 15 min at 37 °C for rat liver ribosomes; buffer D, for 30 min at 24 °C for *A. salina* ribosomes) in the absence and in the presence of RIPs. At the end of incubations, 5 μl portions were withdrawn and appropriately diluted for the assays of protein synthesis. To the bulk of the samples, 1 vol. of cold ethanol was added and, after 10 min at −80 °C, the ethanol-soluble fractions were recovered by centrifugation. Free adenine present in the ethanol-soluble fraction was converted into its etheno derivative by the method of McCann *et al.* (1985): 0.2–0.3 ml portions of the ethanol-soluble fractions were each diluted to 1 ml with water, 0.4 ml of a mixture of 0.14 M-chloroacetaldehyde and 0.1 M-sodium acetate buffer, pH 5.1, was added to each and the samples were heated in a water bath at 80 °C for 40 min. Fluorescence was measured in an Aminco–Bowman spectrophotofluorimeter. Excitation and emission wavelengths were set at 280 and 400 nm respectively.

Each experiment included a standard of adenine in the appropriate buffer and internal standards obtained by adding known amounts of adenine to the ethanol-soluble fractions from control and RIP-treated ribosomes. The amount of adenine released from RIP-treated ribosomes was calculated from the standards after subtraction of the fluorescence reading given by control ribosomes.

H.p.l.c. determinations. After reaction of the ethanol-soluble fractions with chloroacetaldehyde as above, the samples were extracted four times with 1 vol. of water-saturated diethyl ether and passed through 0.45 μm-pore-size filters; 100 μl portions were then analysed with a Waters high-pressure liquid chromatograph equipped with a Waters 740 data module, a model 501 solvent-delivery system, a Lambda-Max model 481 spectrophotometer (set at 254 nm), a model 820-FP Jasco intelligent fluorescence detector (excitation, 315 nm; emission, 415 nm) and a model U6K injector. The column (0.39 cm × 30 cm) was a reversed-phase μBondapak C_{18} (particle size 10 μm) purchased from Waters Associates. It was eluted isocratically with 50 mM-ammonium acetate buffer (pH 5)/methanol (89:11, v/v) at room temperature.

Protection of ribosomes from ricin by adenine. The protective effect was assayed both in the unfractionated rabbit reticulocyte lysate and in the poly(U)-dependent *A. salina* protein-synthesizing system. Adenine was added at the final concentration of 1.6 mM. At this concentration free adenine had no effect on protein synthesis in the absence of ricin.

Equilibrium dialysis. Experiments were performed as previously described (Sperti & Montanaro, 1968), except for the volume of the samples, which was 0.2 ml. The concentration of ricin A-chain was 18 μM and that of [^{14}C]adenine (diluted with unlabelled carrier to a specific radioactivity of 5.4 mCi/mmol) was in the range 10–40 μM. The buffer was buffer D.

RESULTS

As shown in Fig. 1, the adenine released from ricin-treated ribosomes can be easily detected by h.p.l.c. Under the conditions of the h.p.l.c. run, the supernatant from control ribosomes (Fig. 1*b*) gives a single fluorescent peak with a retention time higher than that of the standard of adenine (Fig. 1*a*). The second fluorescent peak that appears after treatment of ribosomes with ricin A-chain (Fig. 1*d*) has a retention time identical with that of the adenine standards (Figs. 1*a* and 1*c*). Identification was confirmed by the addition of authentic adenine to the ricin-treated sample (Fig. 1*e*).

It must be pointed out that the amounts of adenine present in the samples analysed in Fig. 1 were not detected by the u.v.-absorption monitor and that conversion of adenine into its etheno derivative is necessary to confer to h.p.l.c. the increased sensitivity necessary for the determination of adenine in the nanogram range (3.8 ng in the adenine standards; 2.2 ng released from the ricin-treated ribosomes). The h.p.l.c. procedure, although essential for the identification of adenine, is, however, time-consuming, and for routine determinations the direct fluorescence reading of the chloroacetaldehyde-reactive material present in the ethanol-soluble fractions from control and RIP-treated ribosomes was preferred.

Table 1 shows that the amount of adenine released from rat liver ribosomes increased with the dose of ricin A-chain, and that pretreatment with KCl/puromycin in

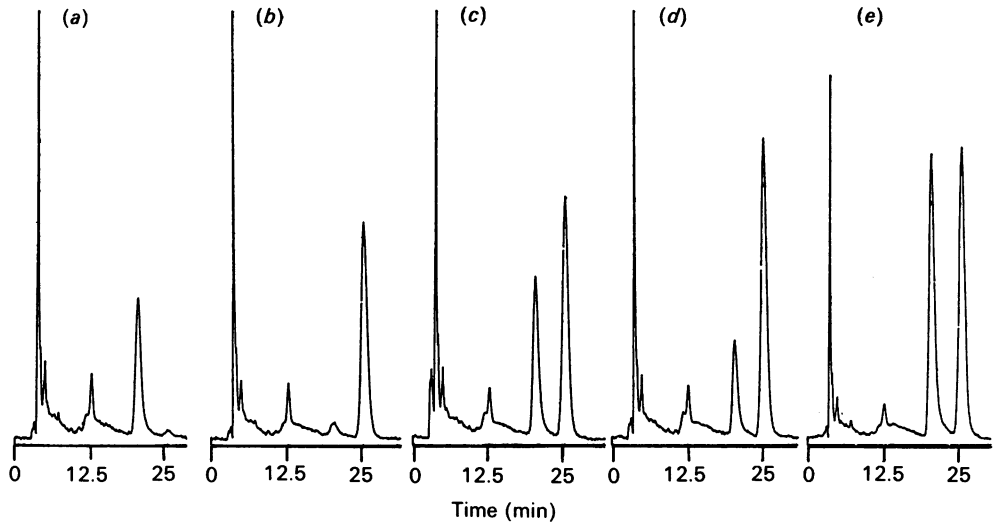

Fig. 1. H.p.l.c. chromatograms for the identification and determination of adenine released by ricin A-chain from rat liver ribosomes

The column was a μBondapak C_{18} and was eluted with 11% (v/v) methanol in 50 mM-ammonium acetate buffer, pH 5.0. The flow rate was 1 ml/min. The fluorescence detector was set at 315 nm excitation and 415 nm emission. The reaction with chloroacetaldehyde was performed as described in the Experimental section in a final volume of 1.4 ml containing: (*a*) 400 pmol of adenine; (*b*) the supernatant from 420 pmol of control ribosomes; (*c*) the supernatant from 420 pmol of control ribosomes + adenine (400 pmol); (*d*) the supernatant from 420 pmol of ricin-A-chain-treated ribosomes; (*e*) the supernatant from 420 pmol of ricin-A-chain-treated ribosomes + adenine (400 pmol). The volume analysed was 100 μl, corresponding to 28.5 pmol of adenine and 30 pmol of ribosomes. The retention time of the adenine peak was 20.5 min and that of the chloroacetaldehyde-reactive material released from both control and ricin-A-chain-treated ribosomes was 25.5 min.

Table 1. Effects of ricin A-chain on protein synthesis by and adenine release from rat liver ribosomes

The experimental procedure is described in the Experimental section. KCl/puromycin pretreatment was performed by incubating 6 nmol of ribosomes in 7 ml of buffer A containing 1 mM-puromycin. After 15 min at 0 °C, followed by 10 min at 37 °C, 1.75 ml portions were pelleted through discontinuous sucrose gradients consisting of 4 ml of 5% (w/v) sucrose in buffer A and 3 ml of 15% (w/v) sucrose in buffer A modified to contain 80 mM-KCl. In protein synthesis, [^{14}C]phenylalanine incorporated in the absence of ricin A-chain was 0.7 pmol/pmol with untreated ribosomes and 1.7 pmol/pmol with KCl/puromycin-treated ribosomes.

Pretreatment of ribosomes	Ricin/ribosome molar ratio	Adenine released (pmol/pmol of ribosomes)	Inhibition of protein synthesis (%)
None	0.09	0.46	35
	0.47	0.59	56
KCl/puromycin	0.10	1.12	36

conditions that dissociate ribosomes into subunits leads to a greater production of adenine.

The same effect after dissociation into subunits (KCl pretreatment) was observed with *A. salina* ribosomes (Table 2). As in the case of rat liver ribosomes, ricin A-chain, at comparable values of polyphenylalanine-synthesis inhibition, released more adenine from the pretreated ribosomes.

Besides ricin A-chain, gelonin also released adenine from both rat liver ribosomes (results not shown) and *A. salina* ribosomes (Table 2). In a previous paper (Brigotti *et al.*, 1989) it was reported that *A. salina* ribosomes are very resistant to gelonin. This resistance can be partially overcome by preincubating ribosomes with the inhibitor before the addition of the components of the protein-synthesizing system. When the ribosomes present in the standard assay of protein synthesis (2.5 pmol) are preincubated with gelonin (in a volume of 20 μl), 50% inhibition is reached at a ribosome/gelonin molar ratio of 1:8 (results not shown). Inhibition is also affected by the concentration of the reactants during preincubation. With a concentration of ribosomes 40-fold higher (5 μM) a 1:0.9 ribosome/gelonin molar ratio gives an inhibition of protein synthesis greater than 50% (see Table 2).

In order to try to establish whether ricin and gelonin are Mg^{2+}-dependent glycosidases, *A. salina* ribosomes were treated with EDTA and then assayed as substrate of RIPs both in the absence and in the presence of added Mg^{2+}. However, as shown in Table 3, the EDTA-treated ribosomes lost irreversibly the ability both to perform protein synthesis and to act as substrate for either ricin or gelonin. The same occurred when the ribosomes were dialysed only against the buffer minus Mg^{2+}, without further addition of EDTA. Very probably, some modification of ribosomes induced by the absence of Mg^{2+} is responsible for the results. Such modification must be extensive, since the amount of chloroacetaldehyde-reactive material released by control ribosomes (incubated in the absence of RIPs) increased considerably after Mg^{2+} depletion (results not shown).

The fluorimetric method will be useful in the study of the kinetics of the enzymic reaction catalysed by ricin and other RIPs. The method, however, cannot be applied to

Table 2. Effects of ricin A-chain and gelonin on protein synthesis and adenine release from *A. salina* ribosomes

KCl pretreatment was performed by suspending 7 nmol of ribosomes in 2.5 ml of buffer B; 1.25 ml portions were then pelleted through discontinuous sucrose gradients consisting of 4 ml of 5% (w/v) sucrose in buffer B and 3 ml of 15% (w/v) sucrose in buffer B modified to contain 120 mM-KCl. During incubation with RIPs, the RIP/ribosome molar ratio was 0.4:1 in the case of ricin A-chain and 0.9:1 in the case of gelonin. In protein synthesis, [^{14}C]phenylalanine incorporated in the absence of RIPs was 4.5 pmol/pmol with untreated ribosomes and 2.5 pmol/pmol with KCl-treated ribosomes.

	Ricin A-chain		Gelonin	
Pretreatment of ribosomes	Adenine released (pmol/pmol of ribosomes)	Inhibition of protein synthesis (%)	Adenine released (pmol/pmol of ribosomes)	Inhibition of protein synthesis (%)
None	0.81	69	0.78	66
KCl	1.15	43	0.95	47

Table 3. Effects of Mg^{2+} and EDTA on the release of adenine from *A. salina* ribosomes by ricin A-chain and by gelonin

The ribosomes used in (*b*) and (*c*) had been extensively dialysed against buffer D minus Mg^{2+}, and then briefly against buffer D minus Mg^{2+} plus 1 mM-EDTA. Control ribosomes, used in (*a*), were dialysed for the same time against standard buffer D (which contains 7 mM-magnesium acetate). During incubation with RIPs, the medium had the same composition as the dialysis buffers, except in (*c*), where it was supplemented with 10 mM-Mg^{2+}.

Incubation conditions		Adenine released (pmol/pmol of ribosomes)		Protein synthesis (pmol of phenylalanine incorporated/pmol of ribosomes)		
EDTA	Mg^{2+}	Ricin	Gelonin	Control	Ricin	Gelonin
(*a*) –	7 mM	0.93	0.88	4.55	0.50	0.80
(*b*) 1 mM	–	0.07	0.07	0.02	0.04	0.01
(*c*) 1 mM	10 mM	0.10	0.00	0.03	0.06	0.03

investigation of the effect of the product of the reaction, adenine itself, since the excess of free base added would mask the amount released by RIPs. Free adenine added to protein-synthesizing systems protects ribosomes from inactivation by ricin; the effect has been consistently observed both with the rabbit reticulocyte lysate system (for example a 60% inhibition by 0.32 nM-ricin becomes 27% in the presence of 1.6 mM-adenine) and with the poly(U)-dependent *A. salina* system (a 60% inhibition by 1.3 nM-ricin becomes 35%).

Although adenine protects ribosomes from inactivation, the ricin-induced release of A-4324 from 28 S rRNA is apparently irreversible, for in the presence of [^{14}C]adenine no radioactivity is incorporated into ribosomes during the course of the reaction. The possibility of an exchange reaction was tested in two systems. One consisted of *A. salina* ribosomes (220 pmol) incubated with ricin A-chain in the presence of a 100-fold molar excess of labelled adenine; incubation was for 1 h at 27 °C. The second contained the rabbit reticulocyte lysate and the assay was performed in the complete protein-synthesizing system. The volume of the standard assay (62.5 μl) was scaled up to 1 ml, [^{14}C]leucine was replaced by the non-radioactive amino acid, and 5 pmol of ricin A-chain and 4 nmol of labelled adenine were present (a 100-fold molar excess with respect to the ribosomes present in the lysate); incubation was for 4 h at 28 °C. In neither case was any radioactivity incorporated in the trichloroacetic acid-insoluble precipitates.

DISCUSSION

The high fluorescence of the 1,N^6-etheno derivatives formed upon reaction of adenine and adenine-containing compounds with chloroacetaldehyde (Secrist *et al.*, 1972) has been applied to the detection of these compounds both on thin-layer and paper chromatograms (Leonard *et al.*, 1972) and in h.p.l.c. (Perret, 1986). Since of the four major bases the reaction with chloroacetaldehyde occurs only with adenine and cytosine and the two etheno derivatives can be easily distinguished by their fluorescence emission maxima (Barrio *et al.*, 1972), the method is particularly suitable for the detection of the adenine released from ribosomes by RIPs. Associated with h.p.l.c., it allows the identification and the quantitative determination of the adenine produced (Fig. 1). More importantly, it can be used by itself for a rapid quantitative determination of the activity of RIPs (Tables 1 and 2).

Under the conditions of the h.p.l.c. run, 5′-AMP, adenine, 3′-AMP and adenosine are eluted from the column in that order. Under the same conditions, the derivatives of 3′-AMP and of adenosine are co-eluted with peak 2 of Fig. 1. This peak, which is present in the

chromatograms from both control and ricin-A-chain-treated rat liver ribosomes, probably results from the activity of contaminant RNAases, which associate with ribosomes during their preparation, producing rRNA fragments, 3′-AMP or even adenosine by the subsequent action of phosphatase(s).

In the direct fluorimetric method, correction for the material released from RIP-treated ribosomes as peak 2 was performed by subtracting from the fluorimetric reading given by RIP-treated ribosomes that obtained from ribosomes incubated in the absence of RIPs. In standard experiments the latter value was fairly constant and approximated that of a standard of adenine equimolar with ribosomes. Only with Mg^{2+}-depleted ribosomes was the value increased, indicating an extensive modification of ribosomes that favours the action of RNAases. The fact that RIPs do not act on Mg^{2+}-depleted ribosomes probably indicates the necessity of this cation for maintaining the secondary ribosomal structure that makes A-4324 a specific substrate for RIPs. Endo *et al.* (1987) showed that this structure is partially lost when RNA is extracted from ribosomes and is completely lost in denatured 28 S rRNA.

The results reported in Tables 1 and 2 show that after pretreatments that dissociate ribosomes into subunits greater amounts of adenine are released at the same dose of ricin A-chain and at comparable values of protein-synthesis inhibition. The adenine released was often even greater than 1 pmol/pmol of ribosomes, suggesting that the pretreatment exposed on ribosomes one or more additional adenine sites that became substrates of RIPs. Since only one 'aniline fragment' has up to now been detected by gel-electrophoretic analysis of rRNA from RIP-treated ribosomes, the hypothesis of small amounts of adenine released from multiple sites appears more likely. The amounts of ricin A-chain used in the present experiments are greater than those used by Endo *et al.* (1988*a*), and a release of adenine from naked rRNA, which is a poor substrate for ricin, can be seen at doses of ricin 10-fold higher than those required for the release from ribosomes (Endo *et al.*, 1988*a*).

Some of the *N*-glycosidases that act on small molecules such as NAD^+, 5′-AMP or purine nucleosides catalyse apparently irreversible reactions (Heppel & Hilmoe, 1952; Tagaki & Horecker, 1957; Hurwitz *et al.*, 1957), whereas others are inhibited by the product of the reaction (McIlwain & Rodnight, 1949; Zatman *et al.*, 1953; Mazelis & Creveling, 1963). The reaction catalysed by RIPs shares both these properties. A possible explanation is a competition between free adenine and A-4324 of 28 S rRNA for the binding site on RIPs. However, a binding of adenine to ricin A-chain was not detected by equilibrium dialysis performed as described in the Experimental section. Thus, if the binding does occur, it must have an association constant lower than 10^3 M^{-1}.

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