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Protein Modification by ADP-ribose via Acid-labile Linkages*

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As substrate for protein-mono-ADP-ribosyltransferases, NAD has been shown to be the donor of ADP-ribose to many different nucleophiles found in proteins. This post-translational modification of proteins has been implicated in the regulation of membrane-associated processes including signal transduction, muscle cell differentiation, and protein trafficking and secretion. Described here is the preparation and chemical characterization of low molecular weight conjugates that were used as models for an acetal linkage between ADP-ribose and the hydroxyl group of a protein acceptor such as serine, threonine, tyrosine, hydroxyproline, or hydroxylysine residues. Model conjugates of ADP-ribose containing an acetal linkage were prepared, their structures were established by NMR, and the chemical stability of the linkage to ADP-ribose was studied and compared to the other known ADP-ribosyl-amino acid linkages. The rapid release of intact ADP-ribose from the acetal model conjugates in 44% formic acid distinguished them chemically from all the other known ADP-ribosyl-amino acid modifications. Rat liver proteins were shown to be modified by ADP-ribose *in vivo* by acid-labile linkages, providing evidence for a new class of endogenous ADP-ribose modification of animal cell proteins. The amount of modification was approximately 16 pmol of ADP-ribose per mg of total protein, and proteins modified by acid-labile linkages were detected in all subcellular fractions examined, suggesting that the scope of this modification *in vivo* is broad.

In eucaryotic cells, NAD is consumed by multiple classes of ADP-ribose transfer reactions involved in the regulation of a variety of metabolic processes (1, 2). Many different cellular nucleophiles have been shown to serve as acceptors of ADP-ribose. Poly(ADP-ribose) polymerase catalyzes transfer of ADP-ribose to glutamate and aspartate (3, 4) residues in proteins and to other ADP-ribose residues to generate polymers of ADP-ribose (1). The synthesis of ADP-ribose polymers is involved in chromatin structural changes necessary for the recovery of cells from DNA damage (1). Endogenous protein-mono-ADP-ribosyltransferases that modify arginine (5–7), cysteine (8–10), asparagine (11), and modified histidine (diphthamide) (12) residues in protein acceptors have been detected. These enzymes have been implicated in the regulation of a number of membrane-associated processes including modulation of adenylate cyclase (13–16), muscle cell differentiation (17–19), and membrane trafficking and secretion (20). NAD glycohydrolases catalyze intramolecular transfer of ADP-ribose to the adenine ring of NAD forming cyclic ADP-ribose and also catalyze hydrolysis of cyclic ADP-ribose to free ADP-ribose (21). Cyclic ADP-ribose has been postulated to be a second messenger involved in the regulation of calcium signaling (22). Free ADP-ribose, which is generated by removal of ADP-ribose from proteins and by the turnover of ADP-ribose polymers and cyclic ADP-ribose, can react nonenzymatically with protein lysine (23) and cysteine (24) residues, resulting in protein glycation.

The versatility of NAD as an ADP-ribose donor in biological chemistry raises the possibility that cellular nucleophiles that heretofore have not been detected may be modified by ADP-ribose. Protein hydroxyl groups have been shown to be the site of numerous post-translational modifications. For example, the regulation of numerous processes is achieved by reversible phosphorylation of protein serine, threonine, and tyrosine residues (25). We describe here the preparation and chemical characterization of low molecular weight conjugates to serve as models for the linkage between ADP-ribose and the hydroxyl group of an acceptor nucleophile in protein. Such linkages are acetals; thus, we have used the term acetal conjugates to distinguish them from the other known classes of ADP-ribose modification of proteins. Information obtained from the characterization of the acetal conjugates has allowed the detection of a new class of ADP-ribose modification of proteins *in vivo* with chemical properties expected for the modification of protein hydroxyl groups. The results described here also suggest that a recently described protein-mono-ADP-ribosyltransferase involved in the regulation of membrane trafficking and secretion modifies protein hydroxyl groups (20).

MATERIALS AND METHODS

Preparation and Characterization of ADP-ribose Acetal Model Conjugates—*Bungarus fasciatus* venom NADase was immobilized on concanavalin A-Sepharose as described previously (26). For preparation of model conjugates, the reaction mixture contained 150 μl/ml immobilized NADase,[1] 2.5 mM NAD, 50 mM potassium phosphate buffer, pH 7.5, 50 mM pyrophosphate, and 250 mM methanol, ethanol, propanol, serine ethyl ester, or threonine methyl ester. The incubation was at 37 °C for 24 h. Aliquots were diluted in 100 mM potassium phosphate buffer, pH 6.0, and subjected to reversed-phase HPLC using a C_{18} column. Isolation of conjugates was by isocratic elution at 1 ml/min with 100 mM potassium phosphate buffer, pH 6.0, containing 5–7% methanol, depending upon the conjugate to be isolated. Preparative amounts of ADP-ribose conjugates were obtained from 50-ml reaction mixtures. The methoxy-, ethoxy-, and propoxy-ADP-ribose conjugates were purified using a preparative C_{18} reversed-phase column with isocratic elu-

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[1] The abbreviations used are: NADase, nicotinamide adenine dinucleotide glycohydrolase; ε-ADP-ribose, 1,N^6-ethenoadenosine diphosphoribose; MOPS, 3-(*N*-morpholino)propanesulfonic acid; HPLC, high performance liquid chromatography; DHB, dihydroxyboronyl.

tion with 50 mM ammonium formate buffer, pH 6.0, containing 5% methanol. The conjugates derived from serine ethyl ester and threonine methyl ester were purified on DHB-Sepharose as described previously (27). Two-ml aliquots of reaction mixture were diluted to 10 ml with 250 mM ammonium formate buffer, pH 8.6. This solution was applied to a previously equilibrated DHB-Sepharose column and washed with an additional 10 ml of buffer followed by 2 ml of water. The conjugates were eluted by two successive additions of 2 ml of ammonium formate buffer, pH 4.6.

A Varian XL-300 NMR spectrometer operating at 299.9 MHz for ^1H and 75.4 MHz for ^{13}C was used to acquire spectral data. Samples were lyophilized three times in D_2O prior to NMR analysis. ^1H NMR spectral parameters were as follows: sweep width, 4400 Hz; data points, 32K; acquisition time, 3.6 s; acquisition delay, 1 s; 32 acquisitions in double precision mode. ^{13}C NMR spectral parameters were as follows: sweep width, 18,000 Hz; data points, 32K; acquisition time, 1 s; acquisition delay, 2 s; 20,000–25,000 acquisitions in double precision mode. ^1H spectra were referenced to HOD at 4.68 ppm, and ^{13}C spectra were referenced using the software of the instrument.

The ^{13}C spectrum of ADP-ribosylthreonine methyl ester showed the following absorptions: δ 176.75, 174.91, 156.39, 152.96, 143.87, 136.83, 122.10, 111.81, 91.10, 87.62, 87.53, 85.18, 85.08, 78.14, 77.86, 74.70, 74.17, 70.71, 69.81, 69.20, 69.17, 64.24, 59.03, and 23.50 ppm. The ^{13}C spectrum of ADP-ribosylserine ethyl ester showed the following absorptions: δ 176.30, 174.89, 172.01, 158.98, 156.37, 152.56, 143.54, 136.82, 122.29, 110.43, 91.11, 87.20, 84.92, 84.83, 78.01, 77.86, 74.71, 74.00, 70.85, 69.19, 67.90, 67.60, 66.57, 66.25, 65.68, 64.14, 64.01, 63.12, 61.28, 60.61, 60.11, 58.63, 58.48, 18.16, and 17.23 ppm. The ^{13}C spectrum of ethoxy-ADP-ribose showed the following absorptions: δ 158.69, 158.66, 155.88, 144.01, 120.00, 109.59, 89.95, 87.01, 86.96, 84.33, 84.28, 77.37, 77.27, 73.90, 73.47, 69.72, 69.67, 68.29, 67.31, and 17.20 ppm.

Chemical Characterizations of ADP-ribose Conjugates—Purified conjugates were incubated in 44% formic acid at 37 °C; 1 M hydroxylamine in 100 mM MOPS buffer, pH 7.0, at 37 °C; 1 M NaOH at 37 °C; or 10 mM $HgCl_2$ at 25 °C. Aliquots were removed and analyzed by C_{18} reversed-phase HPLC. Isocratic elution was done with 100 mM potassium phosphate buffer, pH 6.0, 5% methanol at 1 ml/min.

Preparation of Rat Liver Protein Fraction—An acid-insoluble fraction from rat liver was prepared by modification of procedures described elsewhere (27, 28). Briefly, 1 g of rat liver was dissolved in 2 ml of ice-cold 88% formic acid and suspended quickly by 3 strokes of a Potter-Elvehjem homogenizer. Precipitation was carried out by adding ice-cold trichloroacetic acid to a final concentration of 20%. Due to the acid lability of the linkages under study, the first two steps were accomplished as rapidly as possible, taking care to ensure that the extracts remained at 0 to 2 °C. No significant amount of ADP-ribose was released from model conjugates under these conditions. The precipitate was collected by centrifugation at 800 × *g* for 10 min. The resulting pellet was washed three times with 20 ml of cold diethyl ether and dried. The dry trichloroacetic acid powder was stored at −15 °C. Trichloroacetic acid-insoluble material derived from 1 g of rat liver was dissolved in 4 ml of 6 M guanidinium chloride, 50 mM MOPS buffer, pH 7.0, with the aid of a Dounce homogenizer. The solution was adjusted to pH 7.0 with 5 N NaOH, and any remaining nucleotides were removed by gel filtration as described previously (28).

Protease Treatment and Molecular Sieve Chromatography of Rat Liver Protein Fraction—The acid-insoluble fraction of rat liver (approximately 30 mg/ml) was dissolved in 6 M guanidinium chloride, 50 mM MOPS buffer, pH 7.0, diluted with an equal volume of 50 mM MOPS buffer, pH 7.0, 20 mM $CaCl_2$, and Pronase E (Sigma) was added to a final concentration of 3 mg/ml. The solution was incubated at 37 °C for 24 h prior to fractionation by gel filtration chromatography. Sephadex G-100 resin (100 ml) was hydrated in 500 ml of 6 M guanidinium chloride, 50 mM MOPS buffer, pH 7.0. The resin was washed twice in batch and equilibrated in a 30 × 5 cm Kontes column with the hydrating buffer. The protease digest was applied to the column at a flow rate of 1 ml/min with the same buffer. Four-ml fractions were collected and analyzed for release of ADP-ribose by 44% formic acid as described below.

Subcellular Fractionation of Rat Liver and Analysis of ADP-ribose Released from Rat Liver Extracts—The preparation of subcellular fractions of rat liver was done as described previously (29). The release of ADP-ribose was accomplished by the addition of an equal volume of 88% formic acid to the sample. After incubation at 37 °C for varying periods of time, samples were diluted 9-fold with 1 M guanidinium chloride, 250 mM ammonium acetate buffer, 10 mM EDTA, pH 9.0 (28). The samples were adjusted to pH 9.0 with concentrated ammonium hydroxide and applied directly to an Econo-column with 0.5 ml of DHB-Bio-Rex 70 resin (27). The column was washed with 5 ml of 6 M guanidinium hydrochloride, 50 mM MOPS buffer, 0.25 M ammonium acetate, pH 9.0, followed by 5 ml of 0.25 M ammonium acetate buffer, pH 9.0. The ADP-ribose was eluted from the column with 1 ml of 0.1 M HCl followed by 3.5 ml of H_2O. This solution was adjusted to a final concentration of 0.25 M in ammonium acetate by the addition of a 1 M ammonium acetate. Chloroacetaldehyde was added to a final concentration of 0.8% (w/v), and the pH was adjusted to 4.5 by the addition of concentrated acetic acid. The sample was incubated at 60 °C for 4 h to form the fluorescent derivative, ε-ADP-ribose (27). The pH was adjusted to 9.0, and the sample was applied to a 1-ml column of DHB-Sepharose pre-equilibrated with 10 ml of 0.25 M ammonium acetate buffer, pH 9.0. The resin was washed with 10 ml of 0.25 M ammonium acetate, pH 9.0, and the ε-ADP-ribose was eluted from the column with 3 ml of 25 mM potassium chloride, 10 mM phosphoric acid. The material eluted was separated by HPLC on a Whatman Partisil-10 SAX column (250 × 4.6 mm) preceded by a guard column (50 × 1.5 mm) containing the same matrix. Quantification was by fluorescence monitoring as described elsewhere (28).

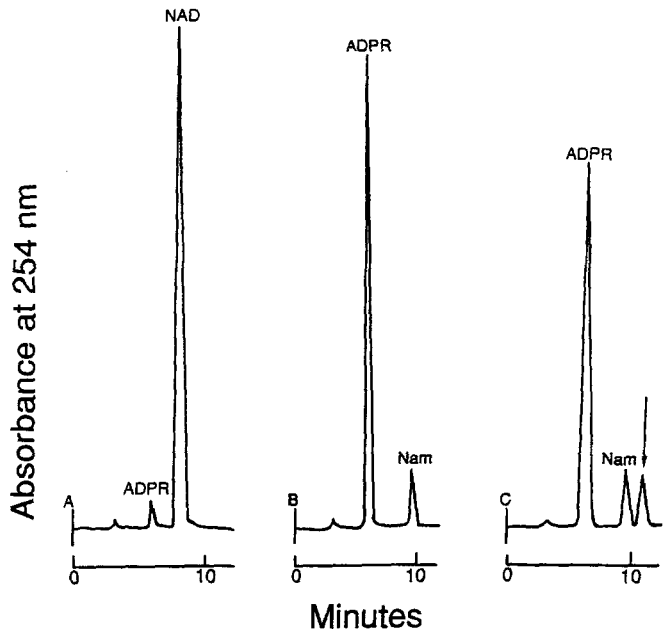

FIG. 1. **HPLC analysis of the reaction of snake venom NADase, NAD, and ethanol.** *A* shows the incubation of NAD and ethanol. *B* shows the incubation of NAD and NADase. *C* shows the complete reaction mixture containing NADase, NAD, and ethanol. The *arrow* indicates the putative model conjugate formed in the reaction. The HPLC running buffer contained 7% methanol.

Protein Analysis—Protein content was estimated by the method of Bradford (30) or by BCA reagent (Pierce Chemical Co.) using bovine serum albumin as a standard.

RESULTS

Preparation of ADP-ribosyl Conjugates Containing an Acetal Linkage—To develop chemical methods for detecting possible ADP-ribose modification of protein hydroxyl groups in the presence of the other known ADP-ribose modifications, we prepared low molecular weight conjugates containing an acetal linkage that could mimic a linkage between the anomeric carbon of ADP-ribose and the hydroxyl group of possible protein acceptors such as serine, threonine, tyrosine, hydroxyproline, or hydroxylysine residues. These model conjugates were prepared using NADase from *B. fasciatus* venom, which previously has been shown by Anderson and co-workers to catalyze a transglycosylation reaction in which ADP-ribose can be transferred to nucleophiles other than water (26). Analysis by HPLC was used to monitor the formation of conjugates. Fig. 1 shows data for the preparation of a conjugate from NAD and ethanol. A series of control experiments were done to demonstrate an enzyme-catalyzed formation of a conjugate between ADP-ri-

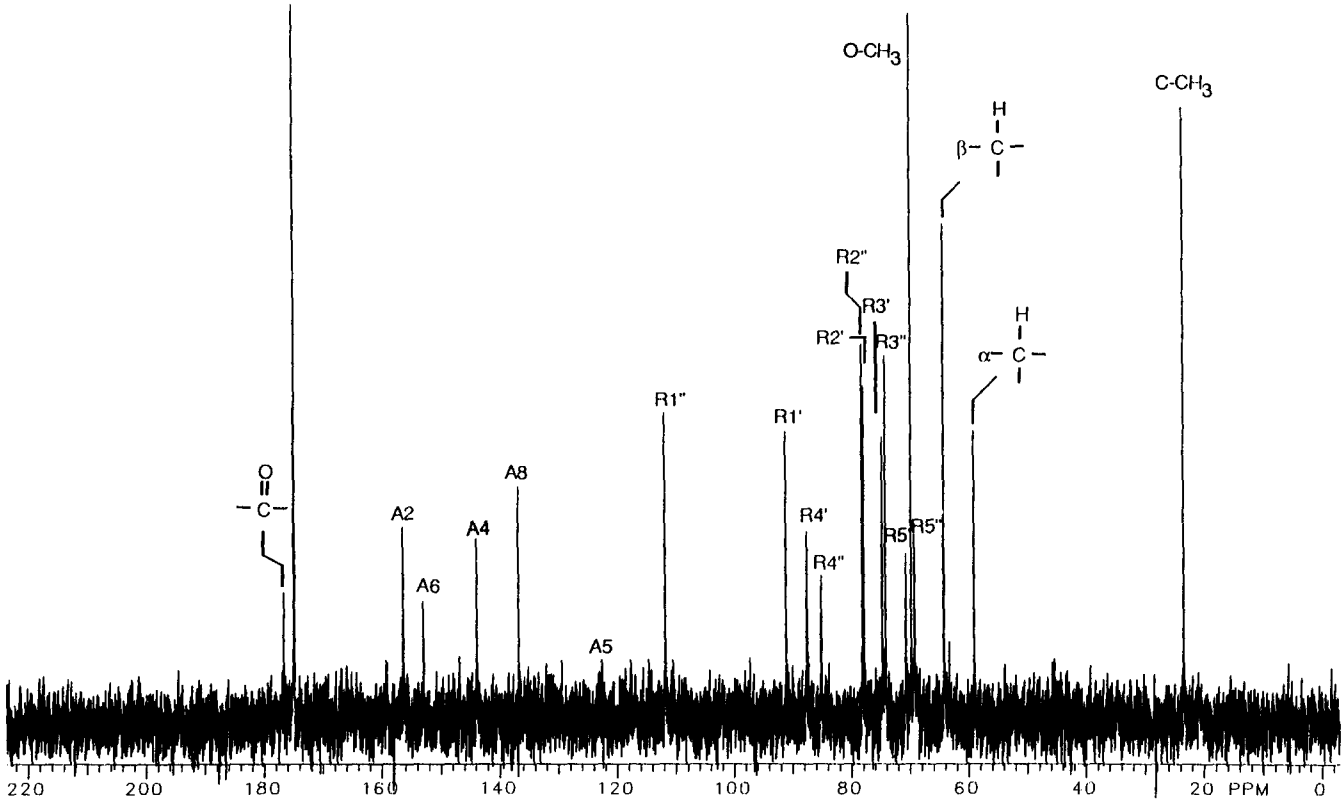

FIG. 2. **^{13}C NMR of the putative ADP-ribosylthreonine methyl ester.** The sample was prepared as described under "Materials and Methods." NMR assignments for ribose and adenine regions are based on published data of related adenosine-containing compounds (32, 33) and on the spectra of the parent compounds. The *A* designations refer to the adenine ring; the *R1'* etc. designations refer to the adenine proximal ribose; and the *R1"* etc. designations refer to the second ribose moiety.

bose and ethanol. The data in *A* show that NAD and ethanol did not react in the absence of NADase, and *B* demonstrates the activity of the enzyme when incubated with NAD alone. *C* shows that a new peak with a retention time of approximately 11 min was observed when the reaction mixture contained NAD, ethanol, and NADase. In a similar manner, putative conjugates were formed using methanol and propanol as ADP-ribose acceptors. The same approach was used to detect putative ADP-ribose conjugates of serine ethyl ester and threonine methyl ester except that an additional control involving the incubation of free ADP-ribose with the amino acid esters was done to check for the formation of ketoamine glycation products resulting from the reaction of ADP-ribose with the free amino group of the amino acids. We have previously described these glycation products and their properties (23). Glycation products were observed, but they could be readily separated from the acetal conjugates because the acetal conjugates are retained on columns of DHB-Sepharose while the glycation products are not retained (23). The acetal conjugates of the amino acid esters were purified on DHB-Sepharose as described under "Materials and Methods."

NMR Characterization of ADP-ribosyl Acetal Conjugates—Structural characterization of the ADP-ribose conjugates was carried out using ^{1}H and ^{13}C NMR. Fig. 2 shows the ^{13}C NMR spectrum of a conjugate of threonine methyl ester and ADP-ribose as an example. With the exception of the signal at 111.81 ppm, the assignments shown were made by comparison with the NMR spectra of the parent compounds. A single set of signals indicated the presence of a single conjugate. The carbonyl signal at 174.91 ppm was due to a small amount of residual ammonium formate in the sample. The signal at 111.81 ppm indicated the presence of the anomeric carbon of an acetal linkage between the 1" carbon of ADP-ribose and the hydroxyl group of threonine methyl ester. In addition, a doublet at 4.8 ppm in the ^{1}H NMR spectrum confirmed the presence of the anomeric linkage (Table I). In contrast to guanidino conjugates of ADP-ribose, which readily anomerize in solution (31), the acetal conjugates cannot form intermediates that could lead to anomerization, thus only one anomeric configuration was present. Analyses of the ethoxy and serine ethyl ester conjugates also confirmed the presence of an acetal linkage involving the 1" carbon of ADP-ribose and the hydroxyl groups of ethanol and serine ethyl ester, respectively. Table I shows the chemical shifts of the ribose-1" carbons and protons and the proton $J_{1''-2''}$ values for each conjugate. The other ^{13}C chemical shifts for these compounds are listed under "Materials and Methods."

The NMR data also allowed determination of the stereochemistry of the glycosidic linkage of the model conjugates. The study of Miwa *et al.* (32) has shown that ^{13}C chemical shift values can unambiguously differentiate the anomeric configuration of an acetal linkage. The signals for α glycosidic linkages occur between 102 and 104 ppm, while β linkages are observed at 109 ppm or higher. The values for the conjugates obtained in this study ranged from 109 to 112 ppm, indicating β configurations. The study of Ferro and Oppenheimer (33) of models of ribosyl acetals showed that the J_{1-2} values from ^{1}H NMR also can be used to assign anomeric configuration. The α configuration shows J_{1-2} values between 4.0 and 4.6 Hz, while the β configuration shows values of 2.2 Hz or less. The $J_{1''-2''}$ for ADP-ribose acetal conjugates were between 1.3 and 1.7 Hz, corroborating a β configuration for the conjugates. The structures of the three conjugates characterized by NMR are shown in Fig. 3. The chemical stability of the methoxy and propoxy conjugates were also studied, but they were not exhaustively characterized by NMR.

TABLE I
Chemical shifts for anomeric protons and carbons and proton $J_{1,2}$ values in D_2O for ADP-ribosyl acetal model conjugates

Acetal mode conjugate	Chemical shift values		$J_{1''-2''}$
	^{13}C	^1H	
	ppm		*Hz*
Ethoxy-ADP-ribose	109.6	4.84	1.7
ADP-ribosylserine ethyl ester	110.4	4.86	1.5
ADP-ribosylthreonine methyl ester	111.8	4.77	1.3

Chemical Stability of ADP-ribosyl Acetal Conjugates—The chemical stability of the model conjugates was studied with the aim of determining conditions that would allow the differentiation of ADP-ribose bound to proteins via an acetal linkage from other known sites of protein modification by ADP-ribose (23). As expected for an acetal linkage, the acetal model conjugates were labile to hydrolysis under acidic conditions. For our studies, formic acid was chosen due to its ability to efficiently solubilize proteins. Fig. 4 shows reversed-phase HPLC analysis following incubation of ethoxy-ADP-ribose in 44% formic acid at 37 °C. *A* and *B* show the reaction at zero time and 30 min, respectively. The released material co-eluted with intact free ADP-ribose, demonstrating that the acetal linkage was the site of cleavage under the acid conditions. Fig. 5*A* shows that free ADP-ribose was released from ethoxy-ADP-ribose with a $t_{1/2}$ value of approximately 19 min. Fig. 5*B* shows the kinetics of release in 44% formic acid for the five acetal conjugates used in this study. All of the conjugates were acid-labile, although the rates of release ranged from $t_{1/2}$ values of 15 to 50 min. In each case, analysis by HPLC confirmed the release of intact ADP-ribose, confirming that the acetal linkage was the site of hydrolysis.

Previously, we have characterized the chemical stability of each of the other protein nucleophiles known to be modified by ADP-ribose (23, 28, 29). In contrast to the acetal model conjugates, ADP-ribose attached to carboxylate (aspartate, glutamate), guanidinium (arginine), sulfhydryl (cysteine), imidazolyl (diphthamide), and amido (asparagine) groups are stable in 44% formic acid at 37 °C, having $t_{1/2}$ values of more than 10 h. Likewise, the ketoamine glycation products formed by ADP-ribose modification of amino (lysine) groups are also stable in 44% formic acid. Thus, the rapid release of intact ADP-ribose from an acetal linkage in 44% formic acid distinguishes the acetal conjugates from all of the other known ADP-ribosyl amino acids. It should be noted that, while the conjugates were rapidly hydrolyzed at 37 °C in formic acid, they were quite stable in ice-cold trichloroacetic acid, with a $t_{1/2}$ value of approximately 4 h. This stability allowed ice-cold trichloroacetic acid to be used for the preparation of tissues as described below.

Further characterizations of the acetal model conjugates demonstrated other differences in chemical stability between these conjugates and other ADP-ribosyl amino acids. Previous studies have shown that the presence of 1 M neutral hydroxylamine results in the release of intact ADP-ribose from guanidinium and carboxylate groups (28) and that 10 mM $HgCl_2$ results in the release of intact ADP-ribose from sulfhydryl groups (29). Fig. 5*A* shows that ethoxy-ADP-ribose was stable to both of these treatments and that the conjugate also was stable to treatment with 1 M NaOH. Similar results were obtained with the other acetal model conjugates (data not shown).

Analysis of Rat Liver Extracts for Endogenous ADP-ribose Modification by Acid-labile Linkages—The rapid release of intact ADP-ribose in 44% formic acid at 37 °C from the acetal model conjugates was used as the basis for searching for proteins modified *in vivo* by linkages with these properties. Trichloroacetic acid-insoluble fractions of rat liver were prepared, solubilized in 6 M guanidinium chloride, 50 mM MOPS buffer, pH 7.0, and subjected to gel filtration chromatography to remove any remaining low molecular weight material that might have remained trapped in the fraction. The high molecular weight fraction was treated with 44% formic acid for 60 min at 37 °C to examine for the presence of ADP-ribose bound by acid-labile linkages. Released material was purified by DHB-Bio-Rex 70 chromatography, converted to an etheno derivative (ϵ-ADP-ribose) by treatment with chloroacetaldehyde, and analyzed by strong anion exchange HPLC (28). Fig. 6*A* shows that material released by formic acid treatment co-migrated with ϵ-ADP-ribose. *B* shows the result when formic acid treatment was omitted from the analysis. *C* shows an analysis in which chloroacetaldehyde was omitted. D shows a chromatogram in which an ϵ-ADP-ribose standard was added to material analyzed as in *A*. Further characterization of the released product by treatment with snake venom phosphodiesterase yielded material that co-migrated with ϵ-AMP (data not shown). These results demonstrated that an acid-insoluble fraction of rat liver contained ADP-ribose bound by acid-labile linkages.

To determine if the ADP-ribose bound by acid-labile linkages was attached to protein, the acid-insoluble fraction from rat liver was treated with proteases and subjected to molecular sieve chromatography using Sephadex G-100 prior to formic acid treatment and analysis for ADP-ribose. The elution profile of a control not subjected to protease treatment is shown in Fig. 7*A*. As expected, the release of ADP-ribose by formic acid was observed only in the fractions corresponding to high molecular weight material. *B* shows that, after protease treatment, only fractions corresponding to low molecular weight material contained ADP-ribose released by formic acid treatment. These data demonstrate that acid-labile ADP-ribose was protein-bound.

The acid-labile ADP-ribose conjugates of rat liver proteins were further characterized by their sensitivity to alkali. For this, the fractions were subjected to treatment with 1 M NaOH for 6 h followed by dialysis prior to treatment with formic acid to release ADP-ribose. Fractions prepared in this manner were compared to fractions that had not been treated with NaOH. The total amount of ADP-ribose released from fractions not subjected to NaOH treatment was approximately 16.0 pmol/mg of protein. Pretreatment with NaOH prior to analysis yielded 8.2 pmol of ADP-ribose/mg of protein, indicating that alkaline treatment had released approximately one-half of the ADP-ribose. To examine this further, the kinetics of release of ADP-ribose by formic acid from the protein fractions was examined (Fig. 8). For proteins not pretreated with NaOH, a biphasic release was observed with a rapid phase having a $t_{1/2}$ of approximately 1.5 min and a slower phase with a $t_{1/2}$ of approximately 20 min. Approximately one-half of the total ADP-ribose released was accounted for in the rapid phase (8 pmol/mg). Alkali-treated proteins showed only a single phase of ADP-ribose release (Fig. 8*B*) with a $t_{1/2}$ of approximately 20 min, corresponding both in stability and amount to the slower phase of acid release seen for proteins not previously treated with NaOH. These data indicate the presence in rat liver proteins of two classes of acid-labile ADP-ribose conjugates. Both classes of ADP-ribose modification were shown to track with rat liver protein in the experiments shown in Fig. 7.

Subcellular Localization of Proteins Modified by ADP-ribose via Acid-labile Linkages—To assess the intracellular distribution of proteins modified by ADP-ribose by acid-labile linkages, homogenates of rat liver were subjected to subcellular fractionation prior to analysis (Table II). The fractions containing the greatest amount of ADP-ribose were the cytoplasmic (47%) and mitochondrial fractions (40%). But, this ADP-ribose modifica-

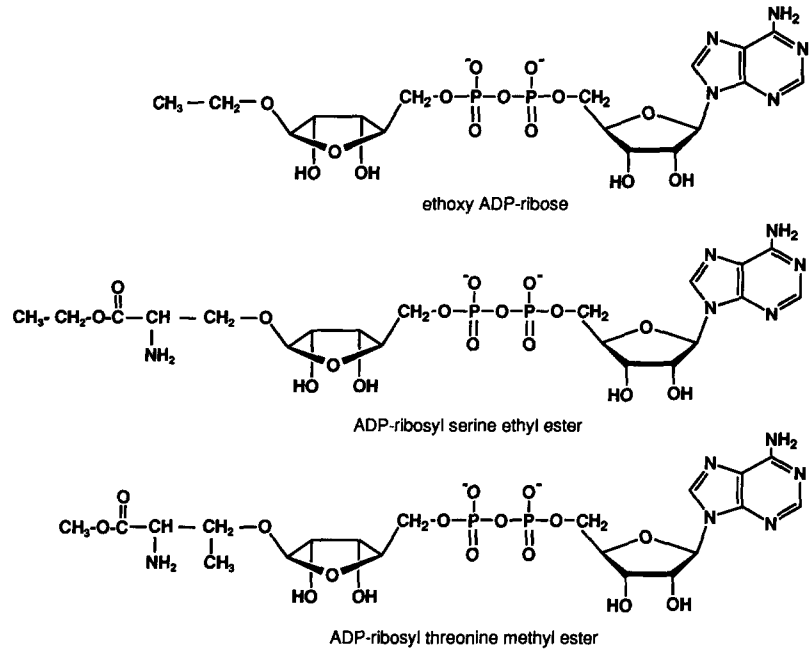

FIG. 3. **Structures of model acetal conjugates synthesized and characterized in this study.**

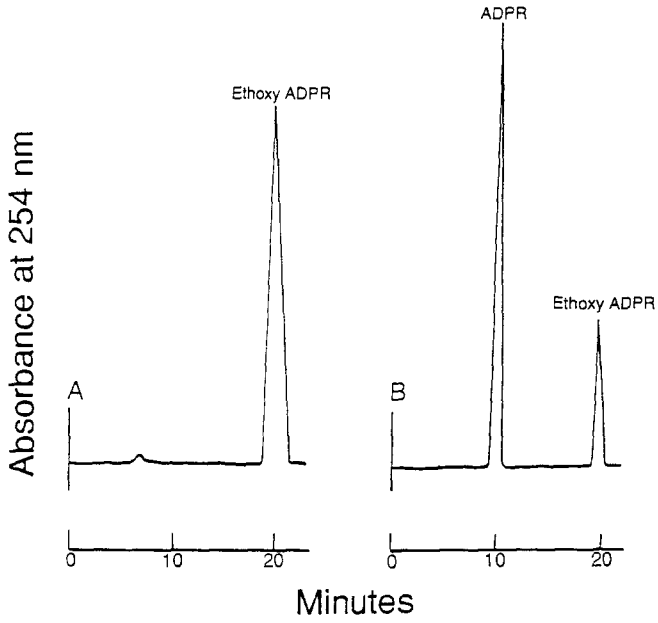

FIG. 4. **Analysis by HPLC of the formic acid-catalyzed hydrolysis of ethoxy-ADP-ribose.** Ethoxy-ADP-ribose was incubated in 44% formic acid at 37 °C. The release products are shown at zero time in *A* and at 30 min in *B*. The HPLC running buffer contained 5% methanol.

tion was detected in all of the fractions examined. The microsomal fraction showed the greatest amount of ADP-ribose on a protein basis with 84 pmol/mg of protein.

DISCUSSION

One approach to the evaluation of possible biological functions of protein modification by monomeric ADP-ribose residues has been to search for and characterize endogenous protein-mono-ADP-ribosyltransferases. Since the identity of most of the endogenous ADP-ribose acceptor proteins is unknown, the ADP-ribosyltransferases have been categorized according to their specificity for the amino acid modified. Endogenous transferases specific for arginine (3, 4), cysteine (8–10), asparagine (11), and diphthamide (12) residues have been described. Both arginine-specific and cysteine-specific transferases have

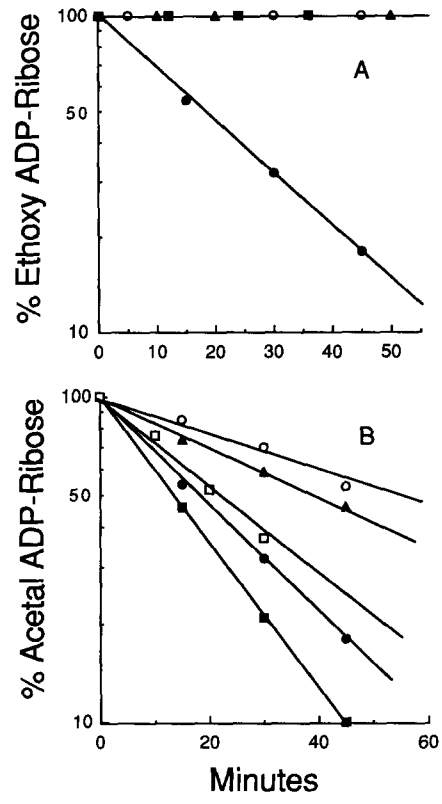

FIG. 5. *A*, stability studies of ethoxy-ADP-ribose. Purified ethoxy-ADP-ribose was incubated in 44% formic acid (●), 1 M hydroxylamine in 100 mM MOPS, pH 7.0 (■), 1 M NaOH at 37 °C (○), or 10 mM $HgCl_2$ at 25 °C (▲). Aliquots were diluted to 1 ml with 50 mM potassium phosphate buffer, pH 6.0. Samples were subjected to reversed-phase HPLC with detection at 254 nm. *B*, kinetics of formic acid-catalyzed hydrolysis of acetal conjugates of ADP-ribose. The purified conjugates were incubated in 44% formic acid at 37 °C. Aliquots were diluted to 1 ml with 50 mM potassium phosphate, pH 6.0. Samples were subjected to reversed-phase HPLC with detection at 254 nm. ▲, methoxy-ADP-ribose; ●, ethoxy-ADP-ribose; ■, propoxy-ADP-ribose; □, ADP-ribosylserine ethyl ester; ○, ADP-ribosylthreonine methyl ester. A representative experiment is shown.

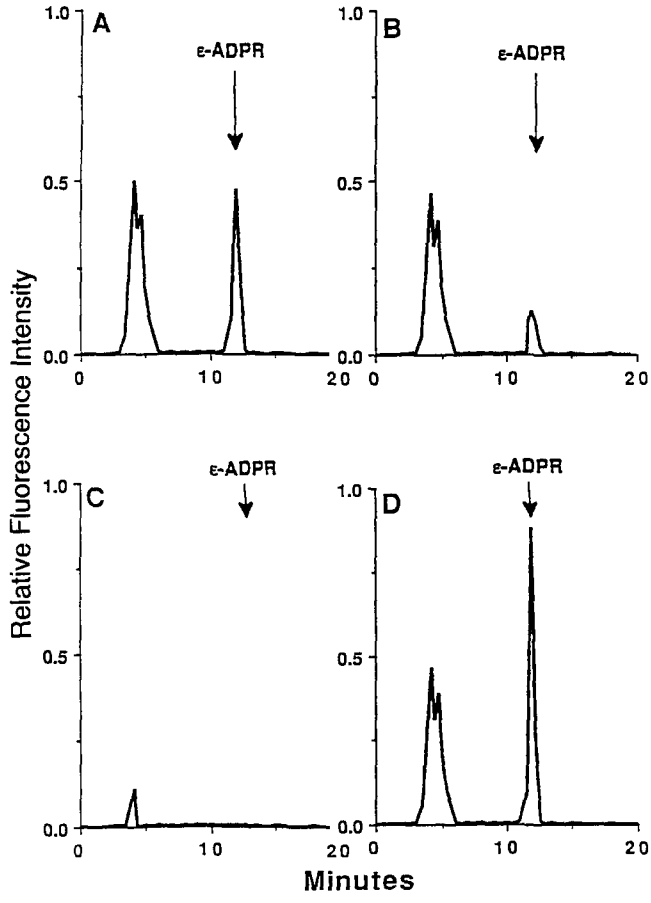

FIG. 6. **Strong anion exchange HPLC analysis of ε-ADP-ribose released by formic acid from alkali-treated rat liver proteins as described under "Materials and Methods."** *A*, formic acid release; *B*, omission of formic acid treatment; *C*, omission of chloroacetaldehyde, which forms the fluorescent derivative of ADP-ribose; *D*, formic acid-treated sample containing a spike of authentic ε-ADP-ribose. The sensitivity of the fluorometer was increased 10-fold at 8 min. *Numbers* on the *ordinate* represent fluorescence intensity after the change in sensitivity. The *arrow* represents the the expected elution position of ε-ADP-ribose.

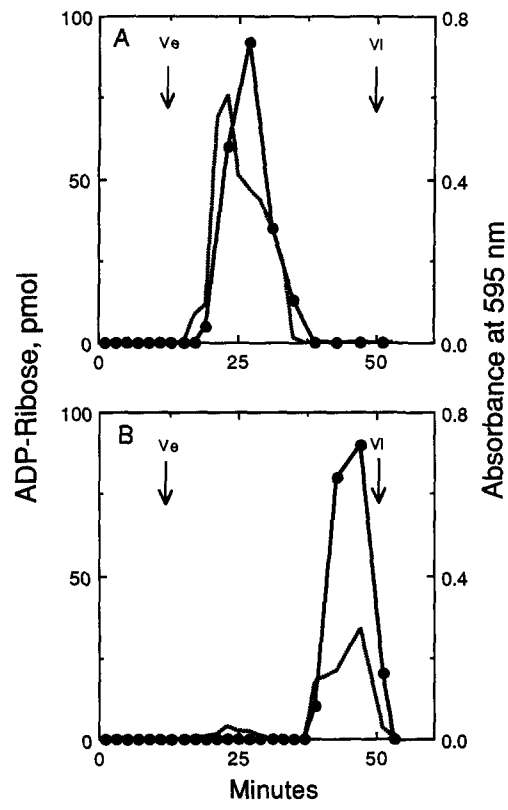

FIG. 7. **Sephadex G-100 molecular sieve chromatography of alkali-treated rat liver proteins.** Fractions were analyzed for ADP-ribose released by acid (*solid line*) as described under "Materials and Methods." The *dashed lines* represent absorption at 595 nm as a measure of protein. *A*, undigested material; *B*, protease-digested material.

been implicated in the regulation of adenylate cyclase activity via ADP-ribose modification of heterotrimeric G proteins (13–16). Arginine-specific transferases also have been implicated in the regulation of muscle cell differentiation (17–19).

Our laboratory has addressed the scope of protein modification by ADP-ribose by examining for the presence of proteins modified by ADP-ribose *in vivo*. This has led to the identification of proteins modified at carboxylate (28), arginine (28), and cysteine (29) residues. The identification of arginine and cysteine residues as sites of ADP-ribose modification was facilitated by the availability of ADP-ribosylating bacterial toxins that allowed the preparation of standards for the development of analytical methods for their detection. The search for endogenous modification by ADP-ribose of protein amino acid residues for which ADP-ribosylating toxins have not been reported has been technically more challenging. For protein hydroxyl groups, we have approached this problem by synthesizing model conjugates to allow characterization of the chemical stability of these linkages. The possibility of an unreported class of ADP-ribose modification of proteins was suggested by an earlier study that identified protein cysteine residues as ADP-ribose acceptors in rat liver proteins (29). In that study, formic acid was used to dissolve a trichloroacetic acid-insoluble fraction of rat liver followed by treatment with mercuric ion to release ADP-ribose from cysteine residues. In those experiments, ADP-ribose was detected following incubation of proteins with formic acid alone, although it was not clear whether this material was derived from a covalent modification or from noncovalently bound ADP-ribose trapped in the protein pellet during precipitation (28). In the experiments described here, care has been taken to exhaustively remove noncovalently bound material prior to analysis.

Characterization of the acetal model conjugates demonstrated that the rapid release of intact ADP-ribose in 44% formic acid distinguishes this linkage to ADP-ribose from all of the other known ADP-ribosyl amino acid linkages (23, 28, 29). This provided the opportunity to search for proteins modified at linkages characteristic of acetals. We initially detected the release of intact ADP-ribose from an acid-insoluble fraction of rat liver which was first subjected to molecular sieve chromatography to remove any ADP-ribose that may have been generated from nucleotides trapped in the acid-insoluble fraction (28). The association of acid-labile ADP-ribose with protein was confirmed by the demonstration that protease treatment resulted in a shift in the elution profile to lower molecular weight fractions (Fig. 7). While the acid-labile ADP-ribose modifications detected have the properties expected for the modification of protein hydroxyl groups, the identification of the amino acid acceptor(s) of ADP-ribose will require further study. Sources of cellular protein hydroxyl groups that could form an acetal linkage with ADP-ribose include serine, threonine, tyrosine, hydroxyproline, and hydroxylysine residues.

Kinetic analysis of ADP-ribose released from protein by formic acid revealed two chemical classes of acid-labile linkages based on the rate of release in acid. One of these classes showed a rate of release ($t_{1/2}$ of approximately 20 min) within the range of release kinetics observed for the acetal model conjugates.

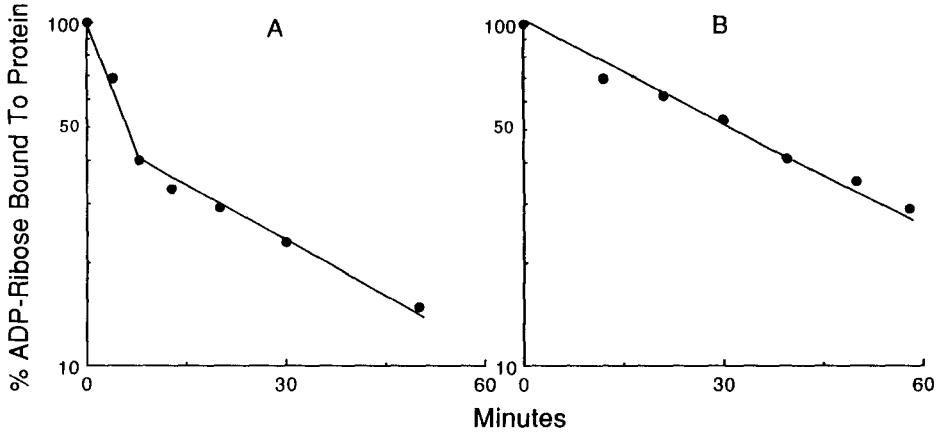

FIG. 8. **Kinetic analysis of the release of ADP-ribose from rat liver proteins.** Sample in *B* was pretreated with NaOH, and the sample in *A* was not treated with NaOH prior to formic acid release of ADP-ribose as described under "Materials and Methods."

TABLE II
Distribution of acid-labile ADP-ribose modified proteins in subcellular fractions of rat liver

Crude homogenate of rat liver was subjected to fractionation and analyzed as described under "Materials and Methods." The values are the mean of triplicate determinations which differed from the mean by less than 10%.

Fraction	Protein	Acid-labile ADP-ribose residues		
	mg	*pmol*	*% total*	*pmol/mg*
Cytoplasmic	291	1943	47	6.7
Mitochondrial	166	1668	40	10
Nuclear	2	41	1	20
Microsomal	5	421	10	84
Plasma membrane	9	109	3	12
Sum of fractions	544	4182	100	7.7
Unfractionated	544	4961		9.1

The second class showed a rate of release more rapid than that of any of the model conjugates. The differences in the rates of release between these two classes in acid may be due to differences caused by the presence of amino acids in proximity to the site of ADP-ribose modification. Although all of the acetal model conjugates released intact ADP-ribose in formic acid, the rate of release varied considerably. The mechanism of hydrolysis of an acetal linkage in acid involves protonation of the oxygen, formation of an oxocarbenium ion intermediate, and hydrolysis of the oxocarbenium ion. The rate-limiting step for hydrolysis may be protonation of the acetal linkage or formation of the oxocarbenium ion intermediate. In the case of the methoxy, ethoxy, and propoxy conjugates, the relative rates of hydrolysis probably reflect the rate of formation of the oxocarbenium ion as the relative rates of hydrolysis agree with the relative stability expected for the respective oxocarbenium ion intermediates. The relative rates of hydrolysis of the amino acid ester conjugates cannot be explained in a similar manner. For these conjugates, the effect of the free amino group on the rate of protonation may account for differences in the rates of hydrolysis. In the proteins analyzed, it seems likely that amino acids in the proximity of the site of modification could increase the rate of protonation and thus result in a more rapid rate of hydrolysis than we observed with the model conjugates. However, in all cases, the hydrolysis results in the release of intact ADP-ribose, demonstrating that the linkage between ADP-ribose and protein is the site of hydrolysis. We have also considered the possibility that the ADP-ribose released by formic acid treatment represents one of the known modifications that is rendered acid-labile by a unique chemistry caused by the proximity of amino acids near the site of modification. This possibility can be ruled out for carboxylate, guanidinium, and sulfhydryl groups modified by ADP-ribose since these modifications are quantitatively removed by 1 M NaOH treatment (23), yet the amount of acid-labile, alkaline-stable modifications remained unchanged. Although this possibility cannot be completely ruled out for imidazolyl and amido linkages to ADP-ribose, it seems unlikely as these linkages are completely stable under a wide range of conditions and we have not detected any release of intact ADP-ribose in formic acid from model conjugates for these linkages (23).

A second possibility is that the classes with different rates of release in formic acid represent ADP-ribose modification of different amino acids. Consistent with this possibility is the fact that these two classes also differed in their sensitivity to alkaline conditions. Glycoproteins have been shown to be glycosylated at serine and threonine residues (34). Also, glucose linkages to tyrosine residues appear to be involved in the priming of glycogen (35, 36). The sugar linkages formed with serine or threonine are alkaline-labile, while the linkages to tyrosine are alkaline-stable (34). The mechanism proposed for the alkaline release involves abstraction of an acidic proton followed by β elimination of the sugar moiety. In ADP-ribose-modified proteins, serine and threonine sites of modification would contain acidic protons making alkaline lability possible while modification at tyrosine, hydroxyproline, or hydroxylysine residues would not. Thus, the rat liver ADP-ribose protein modifications with different rates of release in acid and different stability to alkaline treatment may represent modifications of different amino acids. Interestingly, a recently described endogenous protein-mono-ADP-ribosyltransferase that is activated by the fungal toxin brefeldin A (20) catalyzes a modification with chemical characteristics similar to the class described here that is released by either acid or alkaline conditions.

Although the acetal model conjugates were quite stable in ice-cold trichloroacetic acid, it is still likely that the amount of endogenous protein modification reported here represents an underestimate, particularly for the ADP-ribose class most rapidly released in acid (Fig. 8). The acid lability of this ADP-ribose modification also complicates the detection of protein-mono-ADP-ribosyltransferases and their substrates since acid precipitation prior to electrophoresis is routinely employed in studies designed to detect these enzymes and the proteins they modify.

The cellular distribution of the modification of proteins *in vivo* by acid-labile linkages was assessed by comparing the total amount of the modification in various subcellular fractions. The total amount of modification, approximately 16 pmol of ADP-ribose/mg of protein, is in a similar range observed for

proteins modified by ADP-ribose on arginine (28) and cysteine (29) residues. Assuming an average molecular mass of 40 kDa and one ADP-ribose modification per molecule, this amount of modification would represent ADP-ribose modification of approximately 1 in 1500 protein molecules. When the proteins modified by ADP-ribose linked via acid-labile linkages were characterized as to their cellular location, a wide distribution was observed. This is in contrast to the distribution of ADP-ribosylcysteine linkages, which were found to be located exclusively in the plasma membrane fraction (29). These results indicate that the scope of ADP-ribose modification of protein via acid-labile linkages is broad.

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