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# Purification of ADP-ribosylated nuclear proteins by covalent chromatography on dihydroxyboryl polyacrylamide beads and their characterization

[boric acid gel/*cis*-diol/poly(ADP-ribose)/histones/nonhistone proteins]

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ABSTRACT **Nuclear proteins modified by mono or poly ADP-ribosylation were selectively isolated and purified by covalent chromatography on a dihydroxyboryl polyacrylamide bead column that specifically interacts with *cis*-diol-containing compounds. From rat liver nuclei that had been incubated with $NAD^+$, histones and some nonhistone proteins were extracted with 0.25 M HCl. Approximately 60% of the ADP-ribose incorporated into 20% trichloroacetic acid-precipitable material was recovered in this extract. The ADP-ribosylated material was then isolated from the extract by covalent chromatography on a borate gel column and further purified by carboxymethylcellulose column chromatography. As judged by electrophoretic mobilities in various gel systems and by amino acid compositions, approximately 50% of the ADP-ribose recovered in the carboxymethylcellulose fractions was associated with several nonhistone proteins with molecular weights of $2–6 \times 10^4$, while 35% and 15% were associated with histones H2B and H1, respectively. Since the average chain length of the polymer bound to any of these proteins was less than two ADP-ribosyl units, the percentage distribution reflects the number of ADP-ribosylated sites rather than the chain length.**

Poly ADP-ribosylation is one of the post-translational modifications of nuclear proteins in eukaryotes (1–5) and has been suggested to be involved in the regulation of various nuclear functions, such as DNA synthesis, histone function, and cell differentiation (6–9).

Since the first report from our laboratory in 1968 (10) that a large part of the ADP-ribose incorporated into $CCl_3COOH$-insoluble material from $NAD^+$ was covalently attached to histones, this unique group of basic proteins has been demonstrated to be the major acceptors of ADP-ribose *in vitro* (11–15) as well as *in vivo* (16–18). Recently some nonhistone chromosomal proteins were also suggested to be ADP-ribosylated (19–21). Definitive characterization of the acceptor proteins, however, has not been successful because purification of these proteins, especially of the nonhistone acceptors, proved to be extremely difficult. The major obstacles in purifying these acceptor proteins were the heterogeneity of acceptors induced by variable chain lengths of the bound polymer and their tendency to aggregate with one another.

Recently we developed covalent chromatography for ADP-ribosylated proteins using a dihydroxyboryl polyacrylamide bead column which was originally used for separation of nucleic acids (22, 23). A highly specific interaction of the borate residue with the *cis*-diol portion of the ADP-ribose bound to proteins in the presence of 6 M guanidine·HCl enabled us to selectively isolate ADP-ribosylated proteins from unmodified proteins irrespective of chain length.

This communication describes a simple and effective purification method for ADP-ribosylated proteins by this covalent chromatography and provides conclusive evidence that several nonhistone proteins are ADP-ribosylated in addition to histones H2B and H1.

## MATERIALS AND METHODS

**Chemicals.** [*Adenine-U*-$^{14}$C]$NAD^+$ (237 Ci/mol) was purchased from the Radiochemical Centre, Amersham; *m*-aminophenyl boronic acid, from Sigma; urea (ultra pure), from Schwarz/Mann; calf thymus histone H2B and Combithek [calibration proteins for sodium dodecyl sulfate ($NaDodSO_4$)/polyacrylamide gel electrophoresis], from Boehringer Mannheim; carboxymethyl (CM)-cellulose and Bio-Gel P-60, from Bio-Rad Laboratories; and Soluene 100, from Packard.

**Preparation of Dihydroxyboryl Bio-Gel P-60.** Bio-Gel P-60 (crosslinked polyacrylamide beads) was initially converted to a hydrazide derivative. Aminophenyl boronic acid was then coupled to the derivative via the acyl azide by the method of Inman and Dintzis (24).

**Preparation of Rat Liver Nuclei,** Nuclei were prepared from livers of Wistar rats weighing 300–400 g by the method of Chauveau *et al.* (25) and stored at −60° until use. In order to prevent proteolysis, 0.4 mM phenylmethylsulfonyl fluoride was added to all buffers (26).

**ADP-Ribosylation of Isolated Nuclei and 0.25 M HCl Extraction.** The reaction mixture contained 0.1 M Tris·HCl (pH 8.0), 10 mM $MgCl_2$, 1 mM dithiothreitol, 0.4 mM phenylmethylsulfonyl fluoride, 0.1 mM [*adenine-U*-$^{14}$C]$NAD^+$ (2 Ci/mol), and rat liver nuclei (480 mg of protein) in a total volume of 100 ml. The reaction was at 15° for 10 min and was terminated by the addition of 11 ml of 2.5 M HCl. After the solution was stirred in an ice bath for 1 hr, it was centrifuged at $12,000 \times g$ for 20 min. The supernatant was then made 20% with respect to $CCl_3COOH$ and the precipitate formed was collected by centrifugation. The precipitate was washed three times with ethyl ether and dissolved in 20 ml of 0.1 M potassium phosphate buffer (pH 6.0) containing 6 M guanidine·HCl, followed by dialysis against 10 mM acetic acid and lyophilization.

**$NaDodSO_4$/Polyacrylamide Gel Electrophoresis.** Two electrophoretic systems were used. One was $NaDodSO_4$/polyacrylamide (15%) slab gel electrophoresis in Tris·HCl buffer by the method of Laemmli (27). Samples were solubilized in 10 mM Tris·HCl (pH 7.5)/1% $NaDodSO_4$/25% glycerol/150 mM 2-mercaptoethanol by incubating in a boiling-water bath for 5 min. Under these conditions, the bond between ADP-ribose and protein was completely cleaved, as judged by a decrease of the $CCl_3COOH$-insoluble radioactivity.

The other system was $NaDodSO_4$/polyacrylamide (10%) disc gel electrophoresis in phosphate buffer by the method of Weber and Osborn (28) with a slight modification of solubilization conditions. Samples were dissolved in 10 mM sodium phosphate

Abbreviations: $NaDodSO_4$, sodium dodecyl sulfate; CM-cellulose, carboxymethylcellulose.

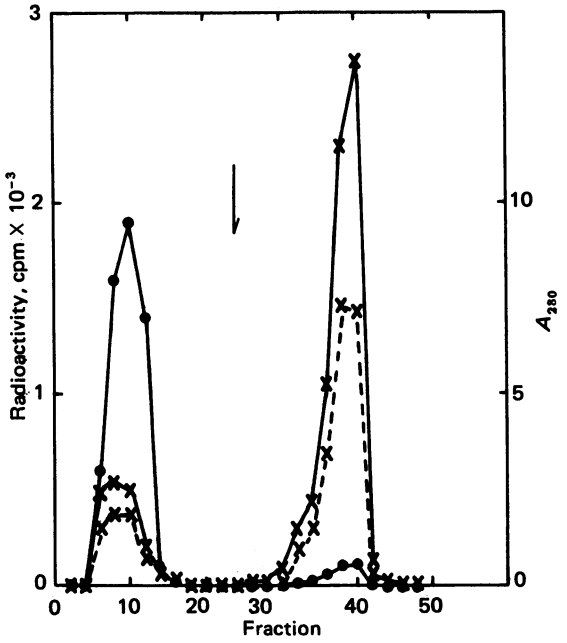


FIG. 1. Dihydroxyboryl Bio-Gel P-60 column chromatography of a 0.25 M HCl extract. Total (×—×) and 20% $CCl_3COOH$-precipitable (×- - -×) radioactivities were determined as described (2). ●—●, Absorbance at 280 nm. The arrow represents the time of the buffer change.

buffer (pH 7.2) containing the same concentrations of $NaDodSO_4$, glycerol, and 2-mercaptoethanol as above and incubated for 3 hr at room temperature. These mild conditions were used in order to prevent nonenzymatic cleavage of the bond between ADP-ribose and an acceptor.

**Acid Urea/Polyacrylamide Gel Electrophoresis.** Polyacrylamide gel electrophoresis was in the presence of 2.5 M urea and 0.9 M acetic acid by the method of Panyim and Chalkley (29).

Preparative gel electrophoresis was performed in a column (1.2 × 13 cm) under the same conditions as above. After electrophoresis at 120 V for 8 hr, a vertical section (0.2 × 13 cm) was cut from the gel and stained with Coomassie Blue. With the stained section as a guide, the desired protein was cut out of the unstained gel and extracted with 0.9 M acetic acid from the gel after maceration. The extract was dialyzed against 10 mM acetic acid and lyophilized.

**Amino Acid Analysis and $NH_2$-Terminal Analysis.** Amino acid compositions of proteins were determined with a JEOL amino acid analyzer (model JLC 6AH) by the method of Spackman *et al.* (30) after hydrolysis with 6.0 M HCl at 110° for 24 hr. The $NH_2$-terminal amino acid was determined by the dansyl technique (31) by use of polyamide thin-layer sheets (5 × 5 cm) for chromatography.

**Other Methods.** The average chain length of poly(ADP-ribose) was determined by an established procedure involving snake venom phosphodiesterase digestion followed by paper chromatography (32). Protein was assayed by the method of Lowry *et al.* (33), with bovine serum albumin as the standard.

## RESULTS

### Purification of ADP-ribosylated nuclear proteins

Poly ADP-ribosylation was carried out with liver nuclei isolated from 20 rats and 0.1 mM radioactive $NAD^+$. Under the conditions used, 1.9 μmol of ADP-ribose was incorporated into 20% $CCl_3COOH$-insoluble material. When the ADP-ribosylated nuclei were treated with 0.25 M HCl, approximately 60% of the ADP-ribose incorporated was extracted along with all of the histones and some nonhistone proteins.

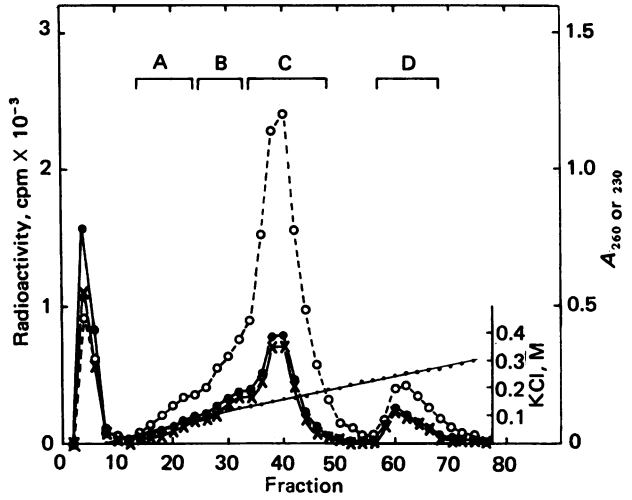


FIG. 2. CM-cellulose column chromatography of the borate column eluate. ○, Absorbance at 230 nm; ●, absorbance at 260 nm; ×, radioactivity;—, KCl concentration.

The lyophilized HCl extract was dissolved in 15 ml of 50 mM morpholine·HCl buffer (pH 8.5) containing 6 M guanidine·HCl. All operations were at 0–4°. After adjustment of the pH to 8.2, the solution was applied to a dihydroxyboryl Bio-Gel P-60 column (1.2 × 22 cm) equilibrated with 50 mM morpholine·HCl buffer (pH 8.2) containing 6 M guanidine·HCl. The column was washed with the same buffer until the absorbance at 280 nm of the eluate decreased to below 0.02. The column was then eluted with 150 mM potassium phosphate buffer (pH 6.0) containing 6 M guanidine·HCl. The flow rate was maintained at 20 ml/hr and fractions of 2.4 ml were collected. Fig. 1 shows the elution profile of the ADP-ribosylated material and protein. More than 95% of the protein passed through the column at pH 8.2, whereas 80% of the radioactivity was adsorbed and eluted by lowering the pH to 6.0. Approximately 60% of the radioactivity eluted was precipitable by 20% $CCl_3COOH$. Some part of the acid-soluble radioactivity probably represents free oligomers and monomers released by nonenzymatic cleavage of the bond between ADP-ribose and an acceptor during chromatography under alkaline conditions. When guanidine·HCl was omitted from the buffer, severe nonspecific interactions occurred among the proteins and also between the proteins and the gel, which interfered with selective adsorption of ADP-ribosylated material to the column. Fractions 32–44 were pooled, dialyzed against 5 liters of 10 mM acetic acid with three changes, and lyophilized.

The lyophilized material was dissolved in 3 ml of 20 mM potassium phosphate buffer (pH 6.0) containing 7 M urea and applied to a CM-cellulose column (1.2 × 5 cm) equilibrated with the same buffer. The column was washed with the equilibration buffer and eluted with a linear gradient of 0–0.4 M KCl contained in the equilibration buffer (total volume, 100 ml). Fractions of 1.4 ml were collected. The elution profile of ADP-ribosylated material is shown in Fig. 2. A large part of the protein applied was adsorbed to the column and eluted in several peaks as the KCl concentration increased. After some proteins were eluted between 0.05 and 0.12 M KCl, the major peak of protein eluted at approximately 0.15 M KCl and a small

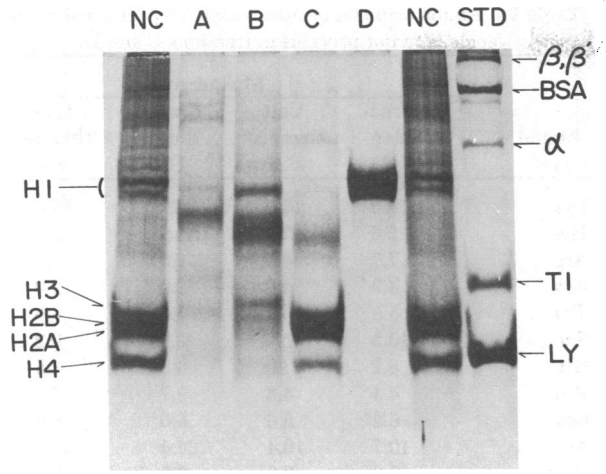


FIG. 3. NaDodSO$_4$/polyacrylamide slab gel electrophoresis of ADP-ribosylated proteins. Fractions A, B, C, and D (9, 16, 20, and 12 μg of protein each) were subjected to electrophoresis after solubilization. Simulataneously, rat liver nuclei (NC), *Escherichia coli* RNA polymerase ($\alpha$, $\beta$, and $\beta$ subunits' molecular weights 3.9, 15.5, and 16.5 $\times 10^4$, respectively), bovine serum albumin (BSA, molecular weight $6.8 \times 10^4$), soybean trypsin inhibitor (TI, molecular weight $2.15 \times 10^4$), and egg white lysozyme (LY, molecular weight $1.4 \times 10^4$) were electrophoresed as the standard proteins. After electrophoresis at 30 mA for 4 hr, the gel was stained with Coomassie Blue.

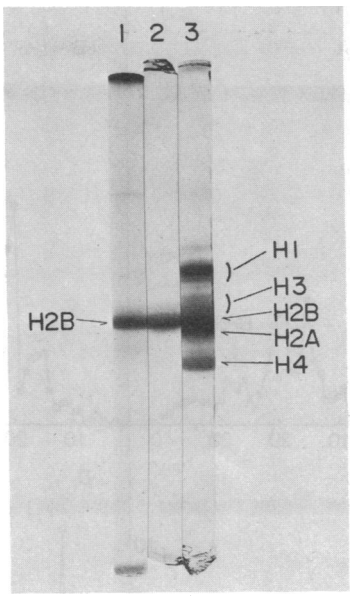


FIG. 4. Acid urea/polyacrylamide gel electrophoresis of fraction C. Fraction C (20 μg of protein) and calf thymus histone H2B were treated with 1 M $NH_4OH$ at 37° for 30 min. After lyophilization, the samples were dissolved in 0.9 M acetic acid containing 3 M urea and, in parallel with untreated whole histone from calf thymus, electrophoresed at 1.5 mA per tube for 6 hr. The gels were stained with Coomassie Blue. *1*, Calf thymus histone H2B; *2*, fraction C; *3*, calf thymus whole histone.

protein peak at 0.25 M KCl. Radioactivity was associated with all protein peaks in complete coincidence with the absorbance at 260 nm. Almost all the radioactivity was precipitable by 20% $CCl_3COOH$, while about 90% of the radioactivity in the unadsorbed fraction was acid-soluble. The radioactive material in the unadsorbed fraction did not penetrate a dialysis membrane with a molecular cutoff of 3500 daltons and interacted less strongly with DEAE-cellulose than free mono- or oligo(ADP-ribose), suggesting that it was not a free monomer or oligomer but was attached to acid-soluble macromolecule(s) containing a positively charged group. When authentic calf thymus histones were chromatographed on a CM-cellulose column under the same conditions as above, histone H1 and the other histones were eluted at KCl concentrations of about 0.25 and 0.15 M, respectively. Fractions 14–24, 25–33, 34–48, and 58–68 were pooled and designated as fractions A, B, C, and D, respectively (Fig. 2). These fractions were dialyzed against 10 mM acetic acid under the same conditions as before. After lyophilization, the materials were dissolved in 1–2 ml of 20 mM potassium phosphate buffer (pH 6.0) and stored at −20°.

## Identification of ADP-ribosylated proteins

Fractions A, B, C, and D were analyzed by NaDodSO$_4$ slab gel electrophoresis (Fig. 3). Unfractionated proteins of rat liver nuclei as well as standard proteins were electrophoresed in parallel slots for references. Under the solubilization conditions used, the bond between ADP-ribose and protein was completely cleaved and acceptor proteins migrated in the gel free from the effect of any ADP-ribose chains that had been attached originally. Fractions A and B contained several nonhistone proteins of molecular weights ranging from 2 to $6 \times 10^4$. Fraction C contained one major protein that had almost the same mobility as that of histones H2A, H2B, and H3 from rat liver and, in addition, contained one minor protein with the same mobility as histone H4. Small amounts of nonhistone proteins were also detected in this fraction. In fraction D, two protein bands were observed, corresponding to subfractions of histone H1.

The proteins in fraction C were further analyzed by acid urea/polyacrylamide gel electrophoresis (Fig. 4). Before electrophoresis, the sample was treated with 1 M $NH_4OH$ at 37° for 30 min to remove the bound ADP-ribose, which affected the mobility of the protein on this gel. As shown in Fig. 4, the major protein migrated with the same mobility as authentic calf thymus histone H2B. Small amounts of histones H3, H2A, and H4 were also detected. Nonhistone proteins, which were observed on an NaDodSO$_4$ gel (Fig. 3), migrated in several faint bands more slowly than H1 in this gel.

The fact that these proteins had been associated with ADP-ribose was ascertained by NaDodSO$_4$ disc gel electrophoresis of each CM-cellulose fraction. Under the solubilization conditions used and these electrophoretic conditions, the bond between ADP-ribose and protein was kept intact. As shown in Fig. 5, radioactivity coelectrophoresed with all visible protein bands except one, histone H4, which migrated slightly faster than the major band in fraction C.

The amino acid compositions and $NH_2$-terminal amino acids of the major proteins in fractions C and D were determined (Table 1). The major protein in fraction C was purified by preparative acid urea gel electrophoresis. This protein had an amino acid composition very similar to that of calf thymus histone H2B and possessed the same $NH_2$-terminal amino acid, proline. The protein in fraction D, which appeared to be a mixture of the subfractions of histone H1, was composed of almost the same amino acids as authentic whole histone H1 of calf thymus, except that tyrosine was not detected in the fraction D material. No $NH_2$-terminal amino acid was detected by the dansyl technique, in agreement with the fact that the $NH_2$-terminus of H1 is blocked by acetylation (36).

These results, together with the electrophoretic analysis shown above, indicate that the major acceptor proteins in fractions C and D were histones H2B and H1, respectively.

The average chain length of the polymer bound to these histone and nonhistone proteins was determined. The average

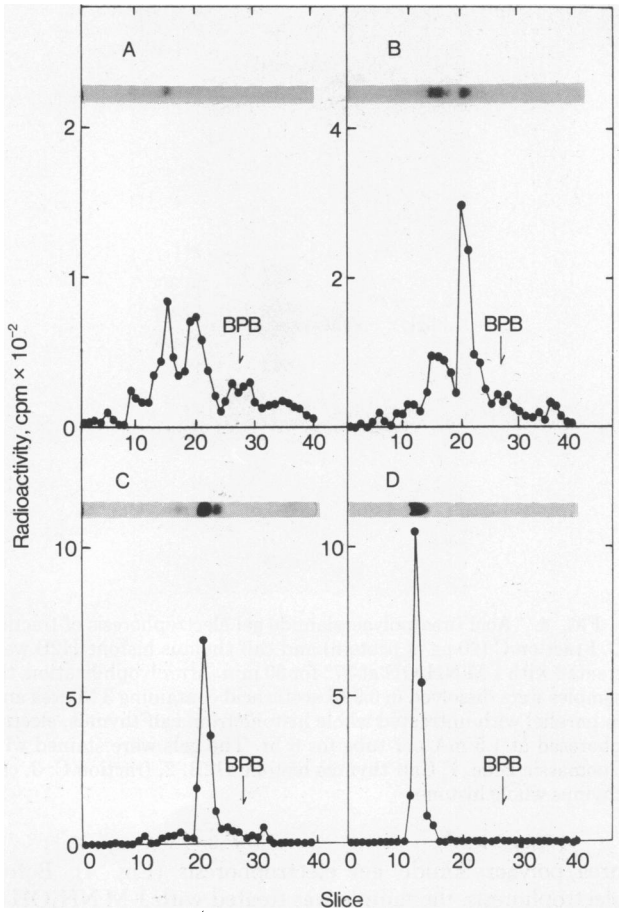


FIG. 5. $NaDodSO_4$/polyacrylamide gel electrophoresis of ADP-ribosylated proteins. Fractions A, B, C, and D were electrophoresed by the method of Weber and Osborn (28) after mild solubilization. After electrophoresis at 6 mA per tube for 6 hr, the gels were cut in half axially. One half was stained with Coomassie Blue and the other half was sliced into 2-mm pieces. The radioactivity was eluted from the pieces with 1 ml of Soluene 100 and measured. (*A*, *B*, *C*, and *D*) Fractions A, B, C, and D, respectively.

chain length in fractions A, B, C, and D were 1.6, 1.4, 1.1, and 1.5 ADP-ribosyl units, respectively.

Quantitative examination indicated that approximately 50% of the acid-insoluble ADP-ribose recovered from the CM-cellulose column was attached to nonhistone proteins and that 35 and 15% were bound to histones H2B and H1, respectively.

## DISCUSSION

In this study, we purified ADP-ribosylated proteins of rat liver nuclei by covalent chromatography on a dihydroxyboryl Bio-Gel P-60 column. This column has various advantages over other purification procedures so far reported: (*i*) a highly specific interaction between the borate residue and *cis*-diol-containing compounds enabled us to selectively isolated ADP-ribosylated proteins from unmodified proteins; (*ii*) differences in the chain length of the bound polymer have little or no effect on the chromatographic behavior of the acceptor protein on the column; and (*iii*) the column works well in the presence of a strong dissociating reagent, such as 6 M guanidine·HCl, which effectively reduces interactions among proteins. Chromatography of ADP-ribosylated material on this column was carried out at a relatively high flow rate because a prolonged exposure of this material to alkaline conditions causes nonenzymatic cleavage of the linkage between ADP-ribose and the acceptors (12).

Table 1. Amino acid compositions and $NH_2$-terminal amino acids of major proteins in fractions C and D

| Amino acid | Mol percent: Fraction C | Calf thymus* H2B | Fraction† D | Calf thymus‡ H1 |
|---|---|---|---|---|
| Lys | 16.0 | 16.0 | 28.9 | 27.9 |
| His | 2.6 | 2.4 | trace | 0.0 |
| Arg | 7.7 | 6.4 | 2.5 | 1.8 |
| Asx | 4.9 | 4.8 | 2.0 | 2.0 |
| Thr | 5.8 | 6.4 | 5.3 | 6.0 |
| Ser | 10.5 | 11.2 | 6.6 | 6.2 |
| Glx | 8.2 | 8.0 | 3.6 | 3.5 |
| Pro | 4.4 | 4.8 | 9.7 | 9.2 |
| Gly | 6.2 | 5.6 | 6.5 | 6.6 |
| Ala | 10.7 | 10.4 | 24.4 | 25.1 |
| Half-Cys | 0.0 | 0.0 | 0.0 | 0.0 |
| Val | 6.9 | 7.2 | 4.4 | 4.6 |
| Met | 1.3 | 1.6 | trace | 0.0 |
| Ile | 4.8 | 4.8 | 1.5 | 1.0 |
| Leu | 5.3 | 4.8 | 4.0 | 4.8 |
| Tyr | 4.0 | 4.0 | 0.0 | 0.5 |
| Phe | 1.7 | 1.6 | 0.5 | 0.5 |
| $NH_2$-terminal | Pro | Pro | ND§ | *N*-Acetylated |

The major protein in fraction C (160 μg), which was purified by preparative acid urea gel electrophoresis, and the protein in fraction D (220 μg) were hydrolyzed with 6 M HCl and subjected to amino acid analyses. For $NH_2$-terminal amino acid analyses, samples of 30 and 50 μg were used, respectively. The serine values were corrected (10%) for hydrolytic losses.

* Calculated from the known sequence (34).
† Average of two experiments.
‡ From Rasmussen *et al.* (35).
§ Not detected.

Analysis of ADP-ribosylated material in the CM-cellulose fractions by various gel electrophoresis techniques and by amino acid determination of certain fractions revealed that several nonhistone proteins were ADP-ribosylated as well as histones H2B and H1. Although small amounts of other histones were detected in the CM-cellulose fraction, evidence for covalent attachment of ADP-ribose to these histones was not obtained. Whether this reflects a very labile bond between these histones and ADP-ribose or indicates a simple contaminant in the ADP-ribosylated proteins is under investigation. The ADP-ribose chains associated with histone H2B appear to be shorter than the others and are almost monomers. Approximately 60% of the H2B molecules recovered were estimated to be associated with radioactive ADP-ribose, assuming that one H2B molecule has only one ADP-ribosylation site. Most of the rest probably represents free H2B molecules that lost ADP-ribose during chromatography by nonenzymatic cleavage of the ADP-ribose–histone bond, although some of the H2B might have been ADP-ribosylated with nonradioactive $NAD^+$ *in vivo*.

It is not likely that all the nonhistone acceptor proteins are proteolytic degradation products of histones because phenylmethylsulfonyl fluoride, an irreversible inhibitor of nuclear serine proteases (26), was used during the early stages of preparation and because some of the nonhistone proteins present have larger molecular weights than histones. One of the nonhistone proteins, which migrated between histones H1 and H2B on a $NaDodSO_4$ slab gel (Fig. 3), may be an acceptor that copurified with poly(ADP-ribose) synthetase, as judged by its electrophoretic mobility on the $NaDodSO_4$ gel (37).

A portion of the ADP-ribose recovered in the flow-through

fraction of the CM-cellulose column appears to be attached to a compound(s) that is not precipitable by 20% $CCl_3COOH$. This compound may be a nucleopeptide reported by Smith and Stocken (20), degradation product of a known protein, or a nonproteinaceous substance.

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