Adenosine Diphosphate Ribose Transferase from Baby-Hamster Kidney Cells (BHK-21/C13)

CHARACTERIZATION OF THE REACTION AND PRODUCT

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Some properties of ADP-ribose transferase, and its reaction product, from BHK-21/C13 cells are described. Enzyme activity was found almost exclusively in nuclei (90%), with the remaining 10% located in the cytosolic fraction. The nuclear enzyme is chromatin-bound and requires bivalent cations, preferably Mg^{2+} , a pH of 8.0 and a temperature of 25°C for optimal activity. Chromatin preparations incorporated radioactivity from [^{14}C]NAD⁺ into acid-insoluble material for about 60 min. Kinetics for substrate NAD⁺ utilization were not of Michaelis–Menten type; biphasic kinetics were shown from a double-reciprocal plot (1/reaction velocity against 1/[NAD⁺]) and from a 'Hofstee' plot (reaction velocity/[NAD⁺] against reaction velocity). The transferase is unstable in the absence of Mg^{2+} ions. It is inhibited by thymidine, nicotinamide and nicotinamide analogues, but not by ATP, which stimulates it at concentrations of 5 mM and above. The enzyme requires thiol groups for activity; it is readily inhibited by *N*-ethylmaleimide at 0.5 mM. The product of the reaction is stable under acid conditions at temperatures up to 25°C, but it is hydrolysed by $HClO_4$ at 70°C. It is resistant to NaOH, but is cleaved from its attachment to protein with alkali into trichloroacetic acid-insoluble and -soluble components. On the basis of Cs_2SO_4 -density-gradient analysis under denaturing conditions (gradients included urea and guanidinium hydrochloride), and analysis of the reaction product directly on hydroxyapatite, we conclude that most of the radioactive ADP-ribose residues are firmly bound to protein, presumably in covalent linkage. Hydroxyapatite-chromatographic analysis of ADP-ribose residues released from protein by alkaline digestion showed a spectrum of molecular sizes including mono-, oligo- and poly-(ADP-ribose). when chromatin was incubated initially with [^{14}C]NAD⁺ for 10 min and then for a further 30 min after addition of excess non-radioactive NAD⁺, only about 10% of the radioactive mono-(ADP-ribose) could be 'chased' into longer-chain molecules. Hydroxyapatite analysis was also used to show that, whereas all ADP-ribose residues were released from protein with NaOH, only 50% of them were susceptible to hydroxylamine. These hydroxylamine-sensitive residues included all size classes, although mono-(ADP-ribose) predominated. Finally, there was an approximately equal distribution of ADP-ribose incorporated into HCl-soluble proteins (including the histones) and HCl-insoluble proteins (including the non-histone proteins) when chromatin was incubated with NAD⁺ up to 0.5 mM, but at higher NAD⁺ concentrations more ADP-ribose was incorporated into the HCl-soluble fraction (82% at 4.0 mM-NAD⁺).

The central role of NAD⁺ is a redox cofactor in cellular metabolism is well understood. Recently,

however, it has become clear that NAD⁺ has another equally important function, namely its involvement as a substrate in the enzyme-catalysed covalent modification of cellular proteins (Hayaishi & Ueda, 1977). In this reaction catalysed by an ADP-ribose transferase, the ADP-ribose moiety of NAD⁺ becomes covalently bound to protein, with

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the elimination of nicotinamide (Hilz & Stone, 1976; Shall *et al.*, 1977).

Two clear examples of the importance of this modification are (1) the ADP-ribosylation and consequent inhibition of elongation factor 1 by diphtheria toxin (Honjo & Hayaishi, 1973) and (2) the modification of *Escherichia coli* DNA-dependent RNA polymerase by an ADP-ribose transferase contained in infecting bacteriophage T4 (Rohrer *et al.*, 1975). In contrast, although ADP-ribosylation was first discovered in chicken liver nuclei (Chambon *et al.*, 1963), the function of this process in eukaryotic cells is poorly understood. This is not surprising, however, since the enzyme activity found in eukaryotic cells modifies a large number of proteins (Rickwood *et al.*, 1977) and can also catalyse the elongation of the first ADP-ribose residues attached to protein, forming poly(ADP-ribose). It seems improbable, therefore, that this modification reaction will have a unique role in cellular metabolism.

In an effort to investigate the possible biological role(s) of this novel process, considerable attention has been focused on the enzymes that catalyse ADP-ribosylation. The nucleus is the predominant location of ADP-ribose transferase (Hilz & Stone, 1976), although some activity has been detected in the mitochondrial (Kun *et al.*, 1975) and cytoplasmic fractions (Roberts *et al.*, 1975). Most investigators have therefore studied the properties of ADP-ribose transferase in preparations of isolated nuclei.

In the present paper we describe some of the fundamental characteristics of the endogenous ADP-ribose transferase, and the reaction product, in chromatin preparations from baby-hamster kidney cells as an adjunct to other studies in this Department concerned with identifying physiological roles for this enzyme (Farzaneh & Pearson, 1978, 1979; Porteous *et al.*, 1979).

Experimental

Materials and methods

With the exception of the items mentioned below, cell reagents were of AnalaR grade, obtained from BDH Chemicals, Poole, Dorset, U.K.

Trizma base, NAD⁺ (grade V), nicotinamide, thymidine, dithiothreitol, Triton N101, ribonuclease A (EC 3.1.27.5), deoxyribonuclease I (EC 3.1.21.1), micrococcal nuclease (EC 3.1.31.1), proteinase K, snake-venom phosphodiesterase (EC 3.1.4.1) and pig brain NAD⁺ glycohydrolase (EC 3.2.2.5) were obtained from Sigma Chemical Co., London S.W.6, U.K.

Poly(ethyleneimine)-cellulose t.l.c. plates were obtained from Macherey-Nagel, Düren, Germany.

Hydroxyapatite was purchased from Bio-Rad Laboratories, Bromley, Kent, U.K.

Cs₂SO₄ (reagent grade) was obtained from BDH, and was recrystallized from ethanol before use.

Nicotinamide-[U-¹⁴C]adenine dinucleotide (ammonium salt; 250 mCi/mmol) was purchased from The Radiochemical Centre, Amersham, Bucks., U.K.

Cells

BHK-21/C13 (Macpherson & Stoker, 1962) cells were grown in monolayer culture at 37°C under 5% CO₂/air in Eagle's medium containing 10% (v/v) calf serum and 10% (v/v) tryptose phosphate broth (Craig & Keir, 1975). The cells were checked regularly for mycoplasma contamination.

Preparation of nuclei

Exponentially growing BHK cells were harvested and washed twice with Tris-buffered saline [consisting of 0.3% (w/v) Trizma base, 0.8% (w/v) NaCl, 0.04% (w/v) KCl, 0.02% (w/v) MgCl₂, 0.05% (w/v) CaCl₂, 0.02% (w/v) Na₂HPO₄ and 0.1% (w/v) glucose; the pH was adjusted to 7.7 at 25°C by the addition of HCl]. All subsequent procedures were carried out at 4°C. The cell pellet from one 80 oz. glass bottle (containing approx. 2 × 10⁸ cells) was resuspended in 8.9 ml of hypo-osmotic buffer (20 mM-Tris/HCl, pH 7.4 at 4°C, containing 2 mM-MgCl₂ and 1 mM-dithiothreitol). After 10 min the swollen cell suspension was sheared by forcible passage through a 21-gauge syringe needle. After addition of 1.75 ml of buffered 2.0 M-sucrose solution (20 mM-Tris/HCl, pH 7.4 at 4°C, containing 2.0 M-sucrose, 2 mM-MgCl₂ and 1 mM-dithiothreitol) the homogenate was vortex-mixed and then centrifuged for 10 min at 800 *g*_{av}. (MSE 2L centrifuge). The supernatant was removed and the nuclear pellet resuspended in 8.0 ml of buffered 0.32 M-sucrose. This crude nuclear suspension was layered over 17 ml of buffered 2 M-sucrose in a 25 ml polypropylene tube and the nuclei were sedimented by centrifuging for 1 h at 4°C and 75000 *g*_{av}. by using a 3 × 25 ml swinging-bucket rotor in an MSE 65 ultracentrifuge.

Preparation of chromatin

The method of chromatin preparation was essentially the same as that of Reeder (1973). All operations were at 4°C. The nuclear pellet from the above procedure was homogenized in a Potter-Elvehjem-type apparatus (1500 rev./min, six strokes) in 10 ml of glycerol buffer [12.5% (v/v) glycerol, 0.5 mM-dithiothreitol, 0.1 mM-EDTA, 10 mM-Tris/HCl (pH 8.0 at 4°C)] and then centrifuged for 15 min at 4°C and 15000 *g*_{av}. by using an 8 × 50 ml fixed-angle rotor in an MSE 18 ultracentrifuge. The supernatant was discarded and the

translucent chromatin pellet resuspended in glycerol buffer (at approx. 2 mg of protein/ml).

Preparation of subcellular fractions

The supernatant from the crude nuclear pellet was transferred to 10 ml centrifuge tubes and centrifuged for 20 min at 4°C and 15000 g_{av} in an MSE 18 high-speed centrifuge by using an 8 × 50 ml fixed-angle rotor fitted with 10 ml adaptors. The pellet was taken as the mitochondrial fraction. The supernatant was again centrifuged for 1 h at 100000 g_{av} by using a 10 × 10 ml angle rotor in an MSE 65 ultracentrifuge. The pellet was taken as the microsomal fraction and the supernatant as the cytosolic fraction.

Assay of ADP-ribose transferase activity

The enzyme preparations (for individual conditions, see the legends to the Figures) were incubated at 25°C in a final volume of 0.2 ml containing 50 mM-Tris/HCl, pH 8.0 at 25°C, 1 mM-dithiothreitol, 5 mM-MgCl₂ and 1 μ M-[¹⁴C]NAD⁺ (250 mCi/mmol). The reaction was terminated by adding 0.2 ml of 10% (w/v) trichloroacetic acid/10 mM-NAD⁺ and the acidified suspension was left at 4°C for 15 min. It was then filtered on glass-fibre discs (Whatman GF/C, 2.3 cm diameter), washed with 2 × 5 ml of cold 5% (w/v) trichloroacetic acid followed by 5 ml of 70% (v/v) ethanol, and then dried. Radioactivity in the discs was determined by liquid-scintillation spectrophotometry by using a toluene-based scintillation fluid [0.4% 2,5-diphenyloxazole and 0.005% 1,4-bis-[5-phenyloxazol-2-yl]benzene] and an Intertechnique SL40 liquid-scintillation spectrometer. The counting efficiency for ¹⁴C samples was 90%.

Snake-venom phosphodiesterase digestion of product

The transferase preparation (approx. 250 μ g of protein) was incubated at 25°C in a final volume of 0.2 ml containing 50 mM-Tris/HCl, pH 8.0 at 25°C, 5 mM-MgCl₂, 1 mM-dithiothreitol and 10 μ M-[¹⁴C]NAD⁺ (250 mCi/mmol). After 30 min the reaction was terminated by adding 0.2 ml of 10% trichloroacetic acid/10 mM-NAD⁺. The acidified suspension was left at 4°C for 15 min and then centrifuged (8000 g_{av} , 2 min) in an Eppendorf centrifuge. The acid-insoluble material was washed with 5% trichloroacetic acid (2 × 0.2 ml) and then 70% ethanol (0.2 ml). It was then dissolved in 0.1 M-NaOH (0.1 ml) and incubated at 37°C for 1 h. After neutralization with 0.1 ml of 0.1 M-HCl, 20 μ l of 0.5 M-Tris/HCl, pH 7.4 at 37°C, 10 μ l of 100 mM-MgCl₂ and 20 μ l of snake-venom phosphodiesterase (1 mg/ml) were added and the mixture was incubated at 37°C for 1 h. Then 25 μ l of proteinase K was added and the mixture incubated for a further

30 min at 37°C. The now-clear solution was concentrated by evaporation with a stream of nitrogen, and a 10 μ l sample was analysed by poly(ethyleneimine)-cellulose t.l.c.

Poly(ethyleneimine)-cellulose t.l.c.

Poly(ethyleneimine)-cellulose plastic-backed plates were washed before use by following the protocol suggested by Randerath & Randerath (1964); other procedures have been previously described (Farzaneh & Pearson, 1978; Stone *et al.*, 1973).

Isolation of the ADP-ribose transferase reaction product

Chromatin (approx. 100 μ g of protein) was incubated at 25°C in a final volume of 0.2 ml containing 50 mM-Tris/HCl, pH 8.0 at 25°C, 5 mM-MgCl₂ and 1 mM-dithiothreitol. The concentration of [¹⁴]NAD⁺ was 1 μ M unless stated otherwise and time of incubation is specified in the legends to individual Figures. The reaction was terminated by adding 0.2 ml of 10% (w/v) trichloroacetic acid/10 mM-NAD⁺ and the acidified suspension left at 4°C for 15 min. The suspension was then centrifuged for 2 min at 8000 g_{av} in a 12 × 1.5 ml fixed-angle rotor in an Eppendorf 5412 centrifuge and the acid-insoluble pellet subsequently washed twice (0.14 ml each) with 5% (w/v) trichloroacetic acid and once with 0.2 ml of 70% (v/v) ethanol. The use of higher concentrations of trichloroacetic acid (up to 20%) did not enhance the recovery of acid-precipitable radioactivity.

Hydroxyapatite analysis of ADP-ribose residues

Hydroxyapatite analysis of ADP-ribose residues was carried out by the method of Tanaka *et al.*, (1977). Hydroxyapatite (0.8 g) was gently resuspended in 1 mM-sodium phosphate buffer (pH 6.8) and left at room temperature for 10 min. The fines were discarded and the remaining suspension was poured into a sealed column (fashioned from a 1 ml plastic disposable syringe) and left to sediment. After 30 min the outlet was opened and the column equilibrated with 1 mM-phosphate buffer (pH 6.8). The acid-insoluble ADP-ribose transferase reaction product was dissolved in 50 μ l of 0.1 M-NaOH and incubated at 37°C for 1 h. After neutralization with 50 μ l of 0.1 M-HCl and addition of 50 μ l of 1 mM-sodium phosphate buffer (pH 6.8), the sample (150 μ l) was applied to the column and eluted with 10 ml of 1 mM-phosphate buffer (pH 6.8); 1 ml fractions were collected under an operating pressure of 15 cm of water. Then a gradient of phosphate buffer (pH 6.8) was applied by using an MSE gradient mixer (type 36657) with 15 ml of 1 mM-phosphate buffer in the mixing chamber and 15 ml of 0.5 M-phosphate buffer in the other chamber. Finally

10ml of 0.5 M-phosphate buffer was applied. The refractive index of each fraction was measured and the phosphate concentration determined by extrapolation from a standard calibration curve. Radioactivity was determined by adding 0.5 ml of each fraction to 0.5 ml of water and 10 ml of toluene/Triton scintillant (Pearson *et al.*, 1976); the samples were then counted in an Intertechnique SL30 or SL40 scintillation spectrometer. All samples were counted until 10000 counts were recorded; the efficiency of counting was 90%.

Cs₂SO₄-density-gradient centrifugation

Cs₂SO₄-density-gradient centrifugation was performed as described by Adamietz & Hilz (1976). Cs₂SO₄ was twice recrystallized from ethanol before use. Approx. 200 µl of the sample to be analysed was added to 4.8 ml of 0.1 M-sodium phosphate buffer (pH 6.0) containing 0.4 M-guanidinium chloride, 6 M-urea, 20 mM-dithiothreitol and Cs₂SO₄ to a refractive index of 1.4137 (25°C). The sample was transferred to a 5.5 ml MSE centrifuge tube, vortex-mixed, and then 0.2 ml of liquid paraffin was layered on top. The tubes were centrifuged for 63 h at 4°C and 130000 *g*_{av.} by using a 6 × 5.5 ml swing-out rotor in an MSE 65 ultracentrifuge. The gradients were fractionated by puncturing the bottom of the tubes and fractions (seven drops each) were collected. Samples were analysed for acid-insoluble radioactivity, refractive index and protein content.

Acid-insoluble radioactivity was measured as previously described (Pearson *et al.*, 1976). The protein content was determined as described by Adamietz & Hilz (1976). Portions (10 µl) were put on cellulose acetate strips. The strips were processed for 20 min in 20% (w/v) sulphosalicylic acid and then in 0.2% (w/v) Coomassie Brilliant Blue R 250 in methanol/acetic acid/water (5:1:5, by vol.). After a washing in ethanol/acetic acid/water (3:1:8, by vol), the remaining spots were cut out, and the protein-bound dye was eluted with 1.0 ml of methoxyethanol and the absorbance measured at 595 nm. There was a linear relationship between absorbance and protein concentration in the range 1–5 µg.

Distribution of ADP-ribose residues in histone and non-histone fractions

Chromatin preparations (0.2 ml, containing 250 µg of nuclear protein) were incubated with 1 µM-[¹⁴C]NAD⁺ as described above. The reaction was terminated by adding 0.2 ml of 0.5 M-HCl and the samples were left at 4°C for 15 min. The HCl-insoluble material was isolated by centrifugation for 15 min at 8000 *g*_{av.} in an Eppendorf 5412 centrifuge. The supernatant was removed and retained. The pellet was resuspended in 0.2 ml of 0.25 M-HCl, by using a motor-driven glass homo-

genizer machined to fit the conical Eppendorf tubes, and re-centrifuged. The supernatant was combined with the first one. The total HCl-soluble fraction was then precipitated by adding an equal volume of 40% (w/v) trichloroacetic acid/20 mM-NAD⁺. The HCl-insoluble fraction was also resuspended in 20% (w/v) trichloroacetic acid/20 mM-NAD⁺ (0.5 ml). Both fractions were filtered on glass-fibre discs (Whatman GF/C, 2.3 cm diameter), which were washed twice (5 ml each) with the trichloroacetic acid solution, once with 5 ml of ethanol and then counted for radioactivity (Pearson *et al.*, 1976).

Protein determination

Protein was determined by the method of Lowry *et al.* (1951), after samples had been freed from interfering substances by precipitation with 5% (w/v) trichloroacetic acid.

Results

Analysis of the ADP-ribose transferase reaction product

In the original studies of ADP-ribose transferase (Doly & Petek, 1966) the reaction products were identified by analysis of the product of digestion with snake-venom phosphodiesterase, 2'-(5-phosphoribosyl)-5'-AMP. For our purposes it was deemed sufficient to demonstrate that (1) the reaction products were uniquely sensitive to snake-venom phosphodiesterase and (2) that the products of the digestion were AMP and 2'-(5-phosphoribosyl)-5'-AMP.

Table 1 shows that the radioactive acid-insoluble material formed after incubation with [¹⁴C]NAD⁺ was insensitive to various hydrolytic enzymes, but was degraded by snake-venom phosphodiesterase. The incomplete release of acid-insoluble radioactivity by the phosphodiesterase could be due to the inaccessibility of the bonds attaching the ADP-ribose units to acceptor proteins (Sugimura, 1973). The

Table 1. *Enzymic sensitivity of acid-insoluble radioactive material isolated from an ADP-ribose transferase incubation*

Incubations were for 1 h at 37°C. The final concentration of all enzymes was 100 µg/ml.

Enzyme	Acid-insoluble radioactivity (c.p.m.)
Control	6814
Deoxyribonuclease I	6737
Ribonuclease A	6925
Micrococcal nuclease	6858
Proteinase K	2961
Snake-venom phosphodiesterase	659
NAD ⁺ glycohydrolase	6730

reaction product was also sensitive to proteinase K, suggesting that the ADP-ribose monomers and oligomers were covalently attached to nuclear proteins.

The snake-venom phosphodiesterase products were separated by poly(ethyleneimine)-cellulose t.l.c.. Only two peaks of radioactivity were obtained, and no radioactivity remained at the origin. One migrated in the same position as a 5'-AMP standard; the other was found to have the same R_F as published for 2'-(5-phosphoribosyl)-5'-AMP (Lehmann *et al.*, 1974). As well as identifying the reaction product, the snake-venom phosphodiesterase digestion also gives valuable information about the average chain length of the ADP-ribose oligomers synthesized. The amount of AMP released determined the number of chains of (ADP-ribose) $_n$. The average chain length can be calculated as follows:

av. chain length =

$$\frac{\text{c.p.m. in AMP} + \text{c.p.m. in 2'-(5-phosphoribosyl)-5'-AMP}}{\text{c.p.m. in AMP}}$$

(Nishizuka *et al.*, 1969)

The average chain length of the (ADP-ribose) $_n$ residues was found to be 2.4 units. It must be stressed that this is only the average chain length of the incorporated radioactive residues. If there are endogenous ADP-ribosylated proteins in the preparation before the addition of [^{14}C]NAD $^+$, then the actual chain length may be quite different; see the Discussion section and also Farzaneh & Pearson (1978).

Characteristics of the chromatin-bound ADP-ribose transferase

The optimum temperature for the reaction of 25°C was established by incubating the transferase preparation at different temperatures and measuring the initial rate of the reaction. In a similar experiment the optimum pH of the reaction was found to be 8.0. The addition of univalent cations (NaCl or KCl) had no effect; the transferase did, however, exhibit a strict requirement for bivalent cation, clearly preferring Mg^{2+} to Mn^{2+} at an optimal concentration of 5 mM. The addition of *N*-ethylmaleimide inhibits the transferase almost completely at 1.0 mM, with 50% inhibition occurring at only 0.1 mM *N*-ethylmaleimide. At these concentrations this compound is specific for thiol groups. We therefore included dithiothreitol at 0.5 mM in all buffers used in the isolation of the ADP-ribose transferase.

Under these optimal conditions, radioactivity from [^{14}C]NAD $^+$ was incorporated into acid-in-

soluble material for about 60 min. On further incubation of the nuclei there was a small loss of incorporated radioactivity, which could indicate either the presence of a hydrolytic enzyme such as poly(ADP-ribose) glycohydrolase (Hilz & Stone, 1976), or the lability of the protein-ADP-ribose linkage at pH 8.0.

The relationship between reaction velocity and substrate concentration is displayed in Figs. 1(a) and 1(b). It is clear that this ADP-ribose transferase preparation displays a biphasic deviation from Michaelis-Menten kinetics. Considerable substrate activation occurs at NAD $^+$ concentrations in excess of 0.15 mM. This behaviour can have diverse origins. Since the enzyme preparation possibly contained endogenous activators and inhibitors, it is difficult to assign a precise reason for the observed substrate activation (see the Discussion section).

In the presence of Mg^{2+} (5 mM) the ADP-ribose transferase is quite stable. However, in the absence of a metal cation activity decreases rapidly at 37°C and at 25°C (Fig. 2a). Fig. 2(b) shows a plot of the

Fig. 1. Relationship between substrate NAD $^+$ concentration and ADP-ribose transferase activity

Chromatin (approx. 100 μg of protein) was incubated for 1 min with the various concentrations of NAD $^+$ shown. Fig. 1(a) shows a double-reciprocal plot (Lineweaver & Burk, 1934) and Fig. 1(b) an Eadie-Hofstee plot (see Hofstee, 1959) of the same data.

Fig. 2. *Stability of the ADP-ribose transferase preparation*

(a) Before determination of ADP-ribose transferase activity, chromatin preparations (approx. 100 μ g of protein) were incubated in either (Δ) 50 mM-Tris/HCl (pH 8.0)/5 mM-MgCl₂ at 37°C, ($\bullet$) 50 mM-Tris/HCl (pH 8.0) at 37°C or ($\circ$) 50 mM-Tris/HCl (pH 8.0) at 25°C. At the times shown samples were assayed (1 min) for ADP-ribose transferase activity. (b) Semi-log plot of the experiment in Fig. 2(a) shown by the filled circles ($\bullet$).

logarithm of the activity remaining at 37°C against time; the curve is clearly biphasic.

The response of the BHK-cell enzyme to various

compounds shows that it behaves very much like the transferase reported in other systems (see Hilz & Stone, 1976). Thus both nicotinamide and thymidine completely inhibited the enzyme at 5 mM, as did the nicotinamide analogue ATDA (2-amino-1,3,4-thiadiazole) at this concentration. At 1 mM these compounds caused 90% inhibition of the enzyme activity. ATP did not inhibit at 1 mM, but did enhance enzyme activity at higher concentrations (5–10 mM) by some 20–30%.

Subcellular location of ADP-ribose transferase activity

Table 2 shows that the nucleus is the major location of ADP-ribose transferase activity in these BHK cells. There was, however, a considerable amount of activity in the cytosolic fraction (about 10%), but, since this was stimulated by the addition of heat-inactivated chromatin preparation, we think it likely that it represents enzyme that is leached from the nuclei during cellular fractionation. The high specific activity found in the microsomal fraction is probably due to microsomal NAD⁺ nucleosidase, which at pH 8.0 catalyses its own ADP-ribosylation (Green & Dobrjansky, 1971).

The nuclear fraction was further subdivided into nucleoplasmic and chromatin fractions. Almost 90% of the nuclear activity appeared to be firmly bound to DNA, and remained in the chromatin fraction (Table 2). This chromatin fraction clearly contained the majority of cellular ADP-ribose transferase activity and was used in all subsequent experiments.

Chemical properties of reaction product

The reaction product is quite stable under the conditions of acid precipitation at 4°C and at 25°C. It is however, like most nucleic acid derivatives, completely hydrolysed by 0.4 M-HClO₄ at 70°C. It is all released from chromatin by mild treatment with alkali, 0.1 M-NaOH at 37°C; of this 40% was trichloroacetic acid-insoluble and the rest was acid-soluble. Since oligo(ADP-ribose) residues were isolated and were found to be resistant to treatment with 0.3 M-NaOH at 37°C for 18 h (results not shown), it is likely that the bond between the first ADP-ribose residue and the acceptor molecule has been hydrolysed.

The products of alkaline hydrolysis were subjected to t.l.c. on poly(ethyleneimine)-cellulose. Fig. 3 shows that most of the radioactivity remained at the origin [the expected position of oligo- and poly-(ADP-ribose)], and 12% migrated with the same R_F as an AMP standard. This presumably results from the alkaline conversion of mono (ADP-ribose) into AMP and gives an indication therefore of the proportion of monomers in the ADP-ribose population (Goebel *et al.*, 1977). The shoulders on the main peak of radioactivity at the

Table 2. *Subcellular distribution of ADP-ribose transferase activity*

Subcellular fractions were assayed for ADP-ribose transferase activity as described in the Experimental section, except that the [^{14}C]NAD $^{+}$ concentration was 2.0 mM. Transferase activity is expressed as nmol of ADP-ribose residues incorporated into acid-insoluble material. Results are the means of three separate experiments (six determinations in total) $\pm$ S.E.M. Symbol * indicates the addition of heat-inactivated nuclei to the assay medium; nuclei (approx. 150 μg of protein) were heated for 15 min at 60°C.

Fraction	Total ADP-ribose transferase activity		Protein content		Specific activity (nmol/min per mg of protein)
	(nmol/min)	(% of total)	(mg)	(% of total)	
Nucleoplasm	10.8 $\pm$ 0.25	10.9	5.3 $\pm$ 0.5	21.5	2.0 $\pm$ 0.14
Chromatin	76.4 $\pm$ 1.0	77.8	7.7 $\pm$ 0.3	31.3	9.9 $\pm$ 0.25
Total nuclear	87.2 $\pm$ 1.0	88.7	12.9 $\pm$ 0.25	52.8	6.7 $\pm$ 0.22
Mitochondrial	0.6 $\pm$ 0.18	0.6	1.6 $\pm$ 0.15	6.5	0.2 $\pm$ 0.05
Microsomal	0.7 $\pm$ 0.16	0.7	0.9 $\pm$ 0.11	3.7	0.8 $\pm$ 0.1
Cytosol	9.7 $\pm$ 1.0	9.9	9.2 $\pm$ 0.35	37.4	1.1 $\pm$ 0.15
*	21.3 $\pm$ 0.32

Fig. 3. Poly(ethyleneimine)-cellulose t.l.c. of the ADP-ribose transferase reaction products after treatment with alkali

Reaction product was incubated for 1 h at 37°C with 0.1 M-NaOH and the products were analysed by poly(ethyleneimine)-cellulose t.l.c. by using the solvent system 0.3 M-LiCl in 0.9 M-acetic acid.

origin probably represent di- and tri-(ADP-ribose) residues.

Covalent linkage to chromatin proteins

Although the oligo- and poly-(ADP-ribose) molecules were only rendered acid-soluble by alkali and hot-acid treatment, it could be argued that the oligo(ADP-ribose)-protein complex was merely an aggregate. This problem was first solved by Adamietz & Hilz (1976), who analysed ADP-ribose-protein complexes by using Cs_2SO_4 -density-gradient analysis under strongly denaturing conditions (6 M-urea, 0.4 M-guanidinium chloride). Fig. 4 shows such an analysis of the BHK-cell chromatin-

bound ADP-ribose transferase reaction product. The close association of much of the radioactively labelled ADP-ribose residues and the protein band is clearly demonstrated (Fig. 4a). Prior treatment with alkali (0.1 M-NaOH, 37°C for 60 min) completely removed all the ADP-ribose from the protein band (Fig. 4b). This result also shows that the residual 40% acid-insoluble material after alkaline treatment described above is due to the intrinsic acid-insolubility of poly(ADP-ribose) and not to the existence of alkali-insensitive (ADP-ribose)-protein linkages. We are uncertain as to why the ADP-ribose residues released from protein with alkali do not band in the gradient, although this might result from diffusion of a population of relatively small molecules.

Size distribution of reaction product

The size distribution of ADP-ribose synthesized *in vitro* was investigated by hydroxyapatite chromatography. Three predominant peaks of radioactivity were detected (Fig. 5), accompanied by many shoulders and small peaks. The first peak (flow-through) should contain AMP and ADP-ribose, and this was demonstrated by poly(ethyleneimine)-cellulose t.l.c. (data not shown). The amount of mono(ADP-ribose) was found to be 9% of the total, and the average chain length of the (ADP-ribose) $_n$ residues was 20.5 units (by using the correlation between phosphate buffer elution and molecular size of ADP-ribose established by Tanaka *et al.*, 1977).

Evidence for two classes of ADP-ribosylation

The synthesis of mono-, oligo- and poly-(ADP-ribose) molecules has been clearly demonstrated. The precise interrelationship between mono- and oligo-(ADP-ribose) was next examined. It seemed

Fig. 4. Cs_2SO_4 -density-gradient centrifugation of the ADP-ribose-protein complex

The transferase product (see the Experimental section) was centrifuged under denaturing conditions (a) after incubation for 1 h at 37°C and pH 6.0 and (b) after incubation for 1 h at 37°C with 0.1 M-NaOH. ●, Radioactivity; ▲, protein (A_{595} ; see the Experimental section); ----, refractive index.

Fig. 5. Hydroxyapatite column chromatography of ADP-ribose residues

Radioactive ADP-ribose-protein material was prepared by incubating chromatin preparations for 30 min with $10\mu\text{M}$ - ^{14}C -NAD⁺ (other conditions are described in the Experimental section). The material was then incubated for 1 h at 37°C with 0.1 M-NaOH, subsequently neutralized with 0.1 M-HCl and applied to the hydroxyapatite. ●, Radioactivity; ----, sodium phosphate concentration.

feasible that eukaryotic cells might contain an ADP-ribose transferase activity similar to that found in T₄ bacteriophages and bacterial toxins, catalysing only the addition of mono(ADP-ribose) residues. In order to investigate this possibility, a 'pulse-chase' experiment was utilized. The synthesis of the (ADP-ribose)_n chains was followed by incubating the transferase for 10 min with ^{14}C -NAD⁺; then an excess of non-radioactive NAD⁺ (4 mM) was added

and the incubation continued for a further 30 min. Another transferase preparation was incubated under identical conditions, but the reaction was stopped at 10 min. Reaction products from both incubations were analysed by hydroxyapatite column chromatography. In the absence of a mono(ADP-ribose) transferase, it is to be expected that all of the radioactivity in the mono(ADP-ribose) peak would move to the position of (ADP-ribose)_n after the

'chase'. In fact, Fig. 6 demonstrates that only 10% of the mono(ADP-ribose) can be 'chased' into oligo- and poly-(ADP-ribose). This result suggests that there may indeed be two distinct classes of ADP-ribosylation in these nuclei, involving the attachment of (1) only monomer ADP-ribose units to protein and (2) monomer units which are subsequently extended covalently into oligo- and poly-(ADP-ribose) molecules. We should add, however, that since the ADP-ribose synthesis occurring in isolated nuclei may be different from that taking place *in vivo* (Adamietz *et al.*, 1978a), the failure of some of the monomeric ADP-ribose to grow during the 'chase' with non-radioactive NAD⁺ may also be due to a non-physiological attachment to acceptor sites not normally utilized *in vivo* which prevents them from being elongated.

Sensitivity of ADP-ribose residues to hydroxylamine

Some investigators, in an attempt to characterize the various modification sites, have used neutral hydroxylamine to distinguish between the protein-ADP-ribose linkages (see Hilz & Stone, 1976). Indeed, Dietrich & Siebert (1974) have suggested that only the mono(ADP-ribose) residues were sensitive to hydroxylamine, whereas the oligo- and poly-(ADP-ribose)-protein linkages were resistant.

In order to investigate which residues are sensitive to hydroxylamine, we devised a separation technique which distinguished between free and bound mono-, oligo- and poly-(ADP-ribose). We had observed that all of the radioactive ADP-ribose transferase product not subjected to alkaline hydrolysis remained firmly bound to hydroxyapatite even after washing the column with 0.5 M-phosphate buffer. No radioactivity above background was

detected in the buffer. After alkali treatment, recovery of ADP-ribose was over 90%. This therefore gave us a method to separate free and protein-bound (ADP-ribose)_n residues.

In this experiment radioactive reaction product was prepared and divided into two fractions of equal volume. One fraction was incubated with 0.1 M-NaOH at 37°C for 1 h; the other was incubated with 0.4 M-hydroxylamine also for 1 h at 37°C. Fig. 7 demonstrates that approx. 56% of the reaction product was sensitive to hydroxylamine. It certainly hydrolyses all the mono(ADP-ribose), but also a proportion of the oligo- and poly-(ADP-ribose) residues are sensitive.

Distribution of ADP-ribose residues in nuclear proteins

Two major nuclear protein classes can be simply separated on the basis of their different solubilities in dilute acid. The basic histone proteins and a few non-histone proteins are soluble in 0.25 M-HCl, whereas the majority of non-histones are insoluble in this. Both protein groups can be precipitated from solution with 20% (w/v) trichloroacetic acid. We have investigated the distribution of ADP-ribose residues, which were synthesized *in vitro*, between the HCl-soluble (including the histones) and HCl-insoluble proteins (including non-histones). Table 3 shows that the proportion of residues linked to the two groups of proteins is about equal, although when the NAD⁺ concentration in the incubation was high (2.0 mM and 4.0 mM) then 70–80% of the ADP-ribose was found in the acid-soluble 'histone'

Fig. 6. Hydroxyapatite analysis of 'pulse-chase' ADP-ribose residues

Chromatin was incubated for 10 min with 1 μ M-[¹⁴C]NAD⁺ (●), or for 10 min with 1 μ M-[¹⁴C]-NAD⁺ followed by a further 30 min incubation after the addition of non-radioactive NAD⁺ to 4 mM (○). In both experiments the radioactive material was incubated for 1 h at 37°C with 0.1 M-NaOH before being applied to the hydroxyapatite. ----, Sodium phosphate concentration.

Fig. 7. Hydroxyapatite analysis of hydroxylamine-sensitive protein-linked ADP-ribose residues

Radioactive ADP-ribose-protein complex prepared in a 30 min incubation of chromatin with 1 μ M-[¹⁴C]-NAD⁺ was incubated either with 0.1 M-NaOH for 1 h at 37°C (●) or with 0.4 M-hydroxylamine for 1 h at 37°C (○). Samples were neutralized before chromatography. ----, Sodium phosphate concentration.

Table 3. *Distribution of ADP-ribose residues in HCl-soluble and HCl-insoluble fractions*

Chromatin was incubated for 30 min with [^{14}C]-NAD $^{+}$ and the HCl-soluble (histone fraction) and HCl-insoluble proteins (non-histones) were isolated as described in the Experimental section.

NAD $^{+}$ (mM)	Radioactivity recovered (% of total)	
	HCl-soluble	HCl-insoluble
0.005	50.8	49.2
0.05	48.2	51.8
0.1	55.8	44.2
0.5	50.9	49.1
2.0	70.4	29.6
4.0	82.4	17.6

fraction. However, a precise interpretation of this intriguing experimental result is not possible at the present time, since the relative solubilities of highly modified histones and non-histones in HCl may differ from their unmodified forms. Further work is needed to obtain a definitive explanation.

Discussion

Properties of the chromatin-bound activity

In general we have found that the chromatin-bound ADP-ribose transferase from BHK cells displays the same properties as transferase preparations from other tissues and cell lines. The low temperature optimum, bivalent-cation requirement and preference for an alkaline pH have been noted by many workers (Stone & Shall, 1973; Brightwell *et al.*, 1975).

As expected, the chromatin-bound transferase was inhibited by nicotinamide and thymidine. Other workers have shown that these inhibitors are highly specific (Preiss *et al.*, 1971; Clark *et al.*, 1971). Indeed, of 11 nicotinamide analogues tested, only 5-methylnicotinamide caused comparable inhibition. In this connection it is noteworthy that the ADP-ribose transferase activity was inhibited by 2-amino-1,3,4-thiadiazole. This was originally described as a niacin antagonist, since its anti-neoplastic and teratological effects could be reversed by the administration of nicotinic acid or nicotinamide (Beaudoin, 1975; Oettgen *et al.*, 1960; Shapiro *et al.*, 1957). It seems likely that 2-amino-1,3,4-thiadiazole disrupts NAD $^{+}$ metabolism in the cell, and our observation suggests that its mode of action may involve the inhibition of ADP-ribose transferase.

There are, however, two features of the chromatin-bound BHK-cell ADP-ribose transferase which contrast with previously published charac-

teristics for this enzyme; these are (1) the relationship between reaction velocity and substrate concentration (Fig. 1) and (2) the heat-inactivation experiment (Fig. 2). These results may be explained by proposing the existence of more than one type of transferase, although different enzymes have not yet been separated chromatographically, or by considering that a single type of enzyme exists in different forms exhibiting different properties. There are a number of precedents for either of these explanations in the literature. For example, Dungan *et al.* (1974) reported distinct differences in the properties of 'soluble' and 'particulate' ADP-ribose transferase preparations from Ehrlich ascites cells. Kidwell & Burdette (1974) showed that the ADP-ribose transferase from S-phase HeLa cells had a higher affinity for NAD $^{+}$ than the preparation from G $_2$ -phase cells. Dramatic differences in substrate kinetics and pH optima have been observed in ADP-ribose transferase preparations from cardiac cells subjected to different growth conditions (Ghani & Hollenberg, 1978). Finally, the purified poly-(ADP-ribose) synthetase from rat liver nuclei does not modify histones in an assay *in vitro*, although histones do activate the enzyme (Okayama *et al.*, 1977). Presumably, therefore, a different enzyme, or a different form of the same enzyme, carries out this function in less-purified systems such as isolated nuclei or chromatin preparations.

Subcellular location of ADP-ribose transferase activity

The early observations of Chambon *et al.* (1966) and Nishizuka *et al.* (1967) indicated that the nucleus was the major intracellular location of ADP-ribose transferase activity. We have found that, although most of the activity resided in the nucleus, a significant amount was also found in the cytosolic fraction. Other investigators (Roberts *et al.*, 1975) have also detected activity in the post-nuclear fraction and have suggested the existence of a cytoplasmic ADP-ribose transferase. It is well established, however, that under the conditions of aqueous subcellular fractionation many enzymes, shown by other techniques to be nuclear in location, appear in the cytoplasmic fraction (Davidson *et al.*, 1958; Behki & Schneider, 1963). The fact that both the cytoplasmic transferase described by Roberts *et al.* (1975) and the one described in our study are stimulated by the addition of heat-inactivated chromatin suggests that the nucleus is the real location. Although the cytoplasmic activity is probably nuclear in origin, it may be a different enzyme from that which remains with the nuclei, since multiple forms of enzymes often differ in their location after subcellular fractionation.

Although the results presented do not preclude the

existence of a cytoplasmic ADP-ribose transferase, presumably modifying cytoplasmic proteins, the main conclusion is that the nucleus is the major location of ADP-ribose transferase activity. It follows, therefore, that ADP-ribosylation must be predominantly involved in nuclear metabolism.

Characterization of the reaction product

It has been demonstrated above that the reaction product of this ADP-ribose transferase preparation is indeed composed of ADP-ribose residues. In the present study Cs_2SO_4 -density-gradient analysis has been used to show that much of the ADP-ribose is covalently attached to protein (Fig. 4a). Some radioactivity in fractions 25–37 appears not to be protein-bound, although we think that it probably is for the following reasons: (1) no ADP-ribose is eluted from hydroxyapatite unless it is first cleaved from protein with alkali (see the Results section on hydroxylamine sensitivity); (2) there is a shift of radioactivity from the top region of the Cs_2SO_4 gradients to positions of greater buoyant density after treatment with alkali, suggesting that (3) at least some of the radioactivity in fractions 25–37 is simply part of the distribution of protein-bound radioactivity at the top of the gradient (this type of distribution occurs when radioactively labelled proteins are centrifuged in caesium salt gradients; C. K. Pearson, unpublished work); (4) our Cs_2SO_4 -gradient profile is comparable with those found by Adamietz & Hilz (1976), who demonstrated by re-banding experiments that this material was in fact protein-bound but possessed a buoyant density greater than that of material at the top of the gradients.

The chemical properties of the ADP-ribose-protein linkage described here are similar to those described by others (Hilz & Stone, 1976). The sensitivity of the long-chain ADP-ribose residues is, however, still controversial. Dietrich & Siebert (1974) maintained that only mono(ADP-ribose) residues were susceptible to neutral hydroxylamine. However, the present studies and those of Adamietz & Hilz (1976) indicate that a proportion of long-chain residues are also susceptible to this reagent. One of the major difficulties in these experiments is the tenacious binding of the long-chain ADP-ribose molecules to nuclear material. To overcome this problem Adamietz & Hilz (1976) separated the released ADP-ribose residues by Cs_2SO_4 -density-gradient centrifugation under denaturing conditions. In our study the free and protein-bound ADP-ribose was separated by hydroxyapatite chromatography, which gave more detailed information about the size of the residues released. It is clear (Fig. 7) that both monomer and polymer residues are hydroxylamine-sensitive, al-

though not all of the oligomeric and polymeric chains are released under these conditions. This could be due to the existence of a different protein-ADP-ribose linkage for these residues, but may also be due simply to steric hindrance. Alternatively, it is possible that ADP-ribose residues become attached to protein in these isolated nuclei in a non-physiological way, which renders some of them susceptible to hydroxylamine. Adamietz *et al.* (1978a), for example, showed that ADP-ribose attached to HeLa-cell histone H1 *in vivo* was insensitive to hydroxylamine whereas 70% of those residues attached to histone H1 in isolated nuclei were released by hydroxylamine treatment. There were a number of differences between the characteristics of the ADP-ribosylation occurring *in vivo* and *in vitro* with the HeLa-cell system, suggesting that results obtained *in vitro* may not reflect the situation *in vivo*. We are unable to comment further on this, since we have not carried out experiments *in vivo* with our BHK-cell system, although since most radioactive ADP-ribose residues (other than monomers) synthesized *in vitro* appear to be attached covalently to chains previously initiated *in vivo* (see below) it seems likely that the protein acceptors for polymeric ADP-ribose found *in vitro* will be the natural acceptors.

There are two well-established methods for determining the chain length of $(\text{ADP-ribose})_n$ molecules synthesized by ADP-ribose transferase preparations. The first method (analysis by snake-venom-phosphodiesterase digestion) depends on the formation of 1 AMP molecule per chain of $(\text{ADP-ribose})_n$ hydrolysed. The total number of ADP-ribose molecules incorporated is known, and therefore the average chain length can be simply calculated from the formula suggested by Nishizuka *et al.* (1969). By using this technique, a wide variety of average chain lengths have been reported, ranging from 1.2 units (Nishizuka *et al.*, 1969), 6 units (Lehmann *et al.*, 1974) to 15–20 units (Hilz *et al.*, 1974). This method, however, only measures the average chain length of the incorporated radioactive residues; the contribution of any endogenous ADP-ribose will not be detected.

In the second method, Sugimura *et al.* (1971) and Tanaka *et al.* (1977) have demonstrated that $(\text{ADP-ribose})_n$ residues can be separated by hydroxyapatite chromatography. This is a physical separation method which depends on the size of the molecule. The average chain length can be calculated from the size distribution of the $(\text{ADP-ribose})_n$ residues.

These two methods will only give the same value if the material labelled *in vitro* is composed entirely of radioactive residues. We demonstrated in our earlier work with *Xenopus laevis* embryonic nuclei that this was the case, and that most of the ADP-ribose

synthesized *in vitro* in this system was a covalent extension of chains previously initiated *in vivo* (Farzaneh & Pearson, 1978). Similar conclusions were also drawn from work in our Department on isolated nuclei from mammalian small-intestinal-epithelial cells (Porteous *et al.*, 1979). The average chain length of the (ADP-ribose)_n synthesized by the BHK-cell chromatin ADP-ribose transferase was found to be 20.5 by using the hydroxyapatite technique and 2.5 by using the phosphodiesterase-digestion method. These results suggest that the majority of incorporation here is also due to the elongation of previously initiated molecules, although about 10% of the chains are newly initiated, as indicated by the proportion of mono-(ADP-ribose) in the hydroxyapatite analysis (see Fig. 5).

The maximum chain length of the BHK-cell ADP-ribose population measured by the hydroxyapatite technique appears to be about 30 residues; however, recent use of gel electrophoresis to separate ADP-ribose chains of different length (Adamietz *et al.*, 1978b; Tanaka *et al.*, 1978) suggests that the hydroxyapatite technique may not be effective for chains longer than about 20 residues (Tanaka *et al.*, 1978).

The present paper concludes our early work on defining some fundamental characteristics of the ADP-ribose transferase system in our BHK-cell line.

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