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DEGRADATION OF $(ADP\text{-}RIBOSE)_n$ IN PERMEABILIZED HELA CELLS

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SUMMARY Determination of $(ADP\text{-}ribose)_n$ degradation rates in permeabilized HeLa cells, measured as loss of acid-insoluble radioactivity from permeabilized cells previously incubated with [^{3}H]NAD^+, showed bi-phasic kinetics. The majority of label was lost within 20 min at pH 6.0 and 37^{o}C and has a half-life of about 12-15 min. The minor ADP-ribose component was either removed very slowly, or appeared to be stable over an 80 min incubation. The degradation rate of the labile component was directly proportional to the initial amount of ADP-ribose present, and was independent of the experimental conditions used to create various elevated levels. The degradation rates of monomeric and oligo/polymeric ADP-ribose were the same, surprising since different enzymes catalyse the respective reactions. The more stable ADP-ribose component could be more inaccessible to degrading enzymes and/or might represent a different linkage to protein, the cleavage of which is slow.

The covalent modification of proteins by ADP-ribosylation is now a well established post-translational modification event. Suggested functions of the process include an involvement in DNA repair activity, cell differentiation and carcinogenesis, although no precise role has yet been defined [1-3].

In order for this protein modification to be important in fine cellular regulatory processes it would presumably have to be readily reversible. A limited number of studies carried out *in vivo* suggest that poly(ADP-ribose) turns over rapidly, usually with a half-life of less than 1 min, with a second component that has a slower turnover rate, $T_{1/2}$ about 5 min [4-6]. This rapid turnover rate apparently contrasts with other reports on work carried out *in vitro* [7,8], including our own previous observations [9,10]. In all cases, however, where a rapid turnover rate has been reported protein

Abbreviations: $(ADP\text{-}ribose)_n$, n=1, monomeric adenosine diphosphate ribose and n>1 for polymer; ADPRT, ADP-ribosyl transferase; DNAase, pancreatic deoxyribonuclease I; DMS, dimethyl sulphate; PMSF, phenylmethylsulphonyl fluoride; PEI-cellulose, polyethylenimine cellulose.

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ADP-ribosylation has been stimulated by treatments which damage DNA, thus promoting ADPRT activity, both in vivo [4-6, 11-13] and in vitro [14].

The simplest interpretation of these observations to date is that the rate of $(ADP\text{-ribose})_n$ degradation is dictated by the amount of ADP-ribose present in the cell [6]. We have tested this proposal using a permeabilized cell system.

MATERIALS AND METHODS

Conditions of HeLa cell growth, cell permeabilization, and ADPRT assays are described in detail elsewhere [15].

ADP-ribose degradation assay. Permeabilized cells were initially incubated for 20 min in the presence of [^{3}H]NAD^+ (see legends) to label protein-bound ADP-ribose residues (conditions as in reference 15). After 20 min the cells ($2x10^6$) were pelleted and resuspended in 300μl of 25mM Na_2HPO_4, 25mM NaH_2PO_4 (pH 6.0) containing 5mM PMSF, 10mM $MgCl_2$, 1mM dithiothreitol and 6mM 3-aminobenzamide. Incubations were then continued at 37°C for up to 2 hours.

The loss of acid-insoluble radioactivity with time was taken as a measure of $(ADP\text{-ribose})_n$ degradation. Control experiments showed that the transferase was completely inhibited under the conditions used.

Hydroxylapatite column chromatography. The sample preparation procedures were as previously described [16]. Briefly, the degradation assays were stopped by adding trichloroacetic acid. The insoluble material was collected and incubated at 37°C with 0.3M NaOH to cleave protein-ADP-ribose bonds and to degrade RNA. DNA and protein were degraded with deoxyribonuclease I and pronase respectively. Samples (500μl) were applied to 1ml hydroxylapatite columns (in a Pasteur pipette) which were then washed with 4.8ml of 1mM potassium phosphate then eluted with a 25ml linear gradient of potassium phosphate buffer (1-500mM, pH 6.8). Fractions of 0.6ml were collected and counted for radioactivity.

RESULTS AND DISCUSSION

Fig. 1 shows the bi-phasic kinetics with which radiolabelled ADP-ribose residues, previously synthesized by incubating permeabilized cells with [^{3}H]NAD^+, are rendered acid-soluble on continued incubation in the presence of 3-aminobenzamide. The ADPRT is completely inhibited under the degradation assay conditions used. In the experiment shown the transferase was activated by incubating the permeabilized cells with various concentrations of DNAase I.

It is clear that the rate of $(ADP\text{-ribose})_n$ degradation over the first 20 min is proportional to the amount present initially. The inset to the figure confirms that the rate at which acid-insoluble radioactvity is lost is linear over this period. Similar experiments (not shown) were carried out in which the initial level of $(ADP\text{-ribose})_n$ was manipulated by incubating cells with

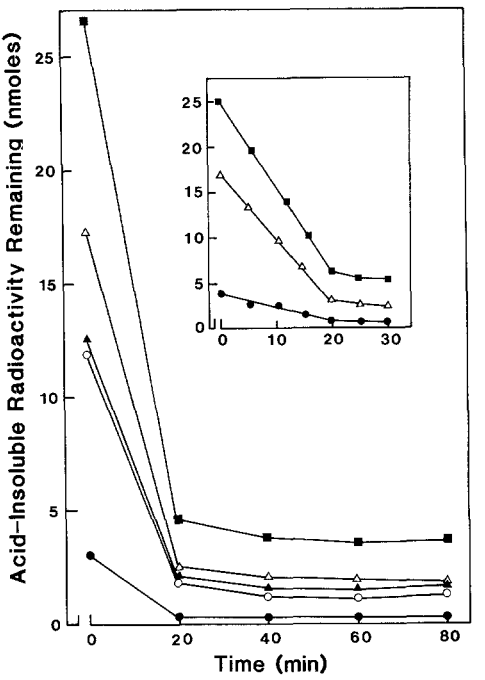

Figure 1. Degradation of ADP-ribose. Permeabilized HeLa cells were incubated for 20 min with 1mM [^{3}H]NAD^+ (5μCi/assay, sp. act. 16.6mCi/mmol) in the absence of pancreatic deoxyribonuclease I (●, control) or with DNAase added to 50μg (○), 75μg (▲), 100μg (△) or 150μg (■) per ml of assay medium. The cells were then pelleted and resuspended in ADP-ribose degradation mix at 37°C containing 6mM 3-aminobenzamide.

The time 0 min (degradation) on the figure refers to the acid-insoluble radioactivity present after the 20 min incubation with the [^{3}H]NAD^+. The inset shows that the rate of degradation was in fact linear over the first 20 min (axes labelling is the same as the main figure).

different concentrations of the transferase substrate, NAD^+, or with dimethyl sulphate, an alkylating agent. The results of all the experiments (Fig. 2) establish that the initial rate of $(ADP\text{-ribose})_n$ degradation is directly proportional to its starting amount and is independent of how this initial quantity is achieved; that is, either with high substrate concentration or by creating DNA damage to stimulate ADPRT activity.

The rate of $(ADP\text{-ribose})_n$ degradation was greater (about 5%) at pH 6.0 than at pH 8.0. Experiments described in this paper were carried out at pH 6.0. Both acidic and alkaline pH optima have previously been reported for various glycohydrolases [17,18] and that of protein lyase, the enzyme catalysing the cleavage of the protein-monomeric (ADP-ribose) linkage, is about pH 7.0 [19,20].

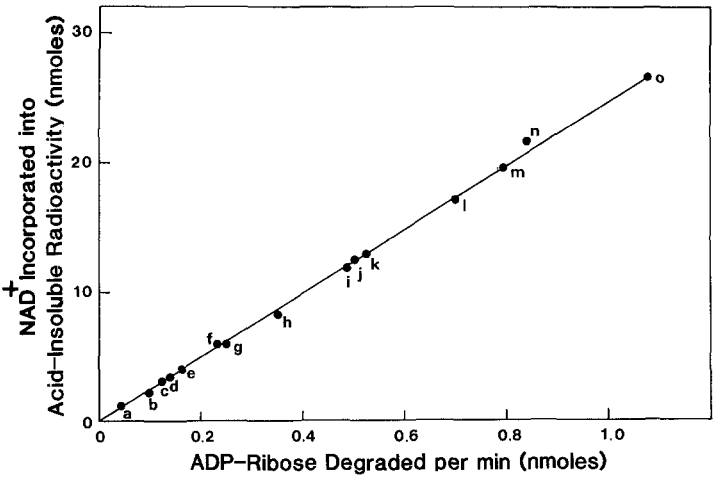

Figure 2. Relationship between the rate of $(ADP\text{-}ribose)_n$ degradation (loss of acid-insolubility over the first 20 min) and the amount of radiolabelled ADP-ribose present at the beginning of the degradation assay.

The following conditions were used during the incubation of the permeabilized cells with [^{3}H]NAD^+ (5μCi/assay):
(a) 0.1mM NAD^+ (b) 0.5mM NAD^+ (c) 1mM NAD^+ (d) 2mM NAD^+ (e) 5mM NAD^+ (f) 25μM DMS, 1mM NAD^+ (g) 100μg/ml DNAase, 0.1mM NAD^+ (h) 100μM DMS, 1mM NAD^+ (i) 50μg/ml DNAase, 1mM NAD^+ (j) 75μg/ml DNAase, 1mM NAD^+ (k) 100μg/ml DNAase, 0.5mM NAD^+ (l) 100μg/ml DNAase, 1mM NAD^+ (m) 100μg/ml DNAase, 2mM NAD^+ (n) 100μg/ml DNAase, 5mM NAD^+ (o) 150μg/ml DNAase, 1mM NAD^+.

In order to confirm that the loss of acid-insoluble radioactivity truly represented $(ADP\text{-}ribose)_n$ degradation, and not that of acceptor proteins, we carried out a double-labelling experiment. Cells were grown in the presence of ^{14}C-labelled amino acids prior to permeabilization to label cell proteins. They were then incubated with [^{3}H]NAD^+ for 20 min and a degradation assay subsequently carried out. It is clear (Fig. 3) that whilst ADP-ribose residues are rendered acid-soluble there is no loss of labelled proteins, provided protease inhibitors are present.

It has previously been considered that the faster turning over component might be degradation of polymeric ADP-ribose, with the lyase catalysed removal of monomeric ADP-ribose from protein acceptors being rate-limiting [4]. Hydroxylapatite analysis of radioactive chains synthesised in the permeabilized cells shows that in our experiments in which increases in substrate concentration were used to elevate ADP-ribose levels, then mostly monomeric ADP-ribose was present (Fig. 4a; monomer = 87% of radiolabelled ADP-ribose, peak at fraction 6). This predominance of monomer under

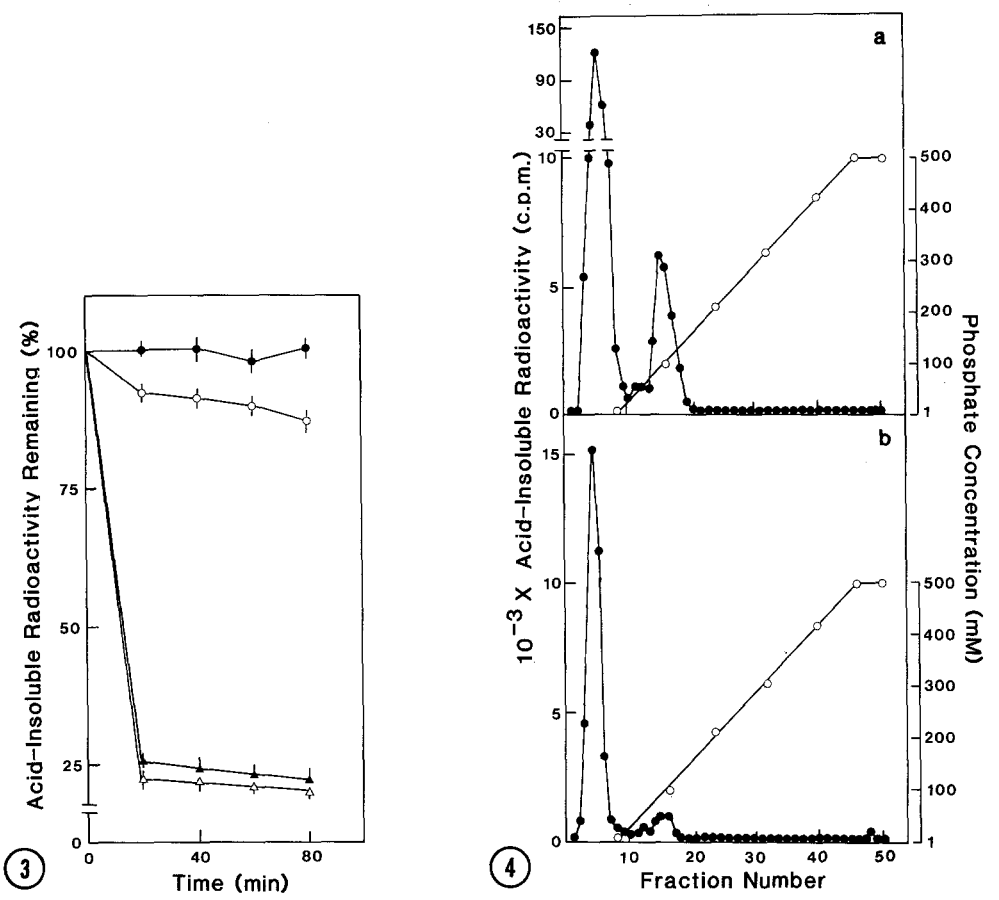

Figure 3. Loss of acid-insoluble radioactivity is due to the degradation of $(ADP\text{-}ribose)_n$ and not protein,. HeLa cells were grown for 24h in the presence of a ^{14}C-labelled amino acid mixture (0.3μCi/ml medium; Amersham mixture CFB 104) to radiolabel proteins. They were harvested, permeabilized and incubated with 1mM [^3H]NAD^+ (sp. act. 16.6mCi/mmole). They were then resuspended and incubated in the degradation assay solution. The Fig. shows the loss of radioactivity when cells were incubated with 0.5mM PMSF throughout the permeabilization, transferase reaction and degradation period (^{14}C,● ; 3H,▲) or without the protease inhibitor (^{14}C,○ ; ^3H,△).

Figure 4. Hydroxylapatite column chromatography of ADP-ribose from permeabilized cells. Exponentially-growing HeLa cells were permeabilized and incubated with 1mM [^3H]NAD^+. They were then resuspended in degradation assay mix and incubated for 0 min (a) or 60 min (b). ADP-ribose chains were subsequently cleaved from proteins with alkali before chromatography. In experiment (a) 259,881 c.p.m. were applied to the column and recovery was 105%. In experiment (b) 45,198 c.p.m. were applied and recovery was 93%. Symbols: (●), radioactivity; (○) phosphate concentration.

conditions in which no DNA damage has been intentionally introduced is consistent with the situation *in vivo* [21,1].

Thin layer PEI-cellulose chromatography was used to confirm the identity of mono(ADP-ribose) eluting from hydroxylapatite in all experiments. When

ADP-ribose content was elevated by DNAase activation of the transferase then additional synthesis of oligomeric and polymeric ADP-ribose became evident (Fig. 5a; monomer = 63% and polymer = 37% of total radiolabel).

During a 60 min degradation assay, therefore, most (90%) of the acid-insoluble radioactivity lost in the first experiment (Fig. 4b) was due to removal of mono(ADP-ribose), presumably catalysed by protein lyase, whereas in the DNAase experiment (Fig. 5b) monomer accounted for 52% and polymer 48% of the radioactivity lost. Despite this different composition of substrates for degrading enzymes (probably lyase and glycohydrolase) a direct relationship is obtained between the initial amount of radiolabelled

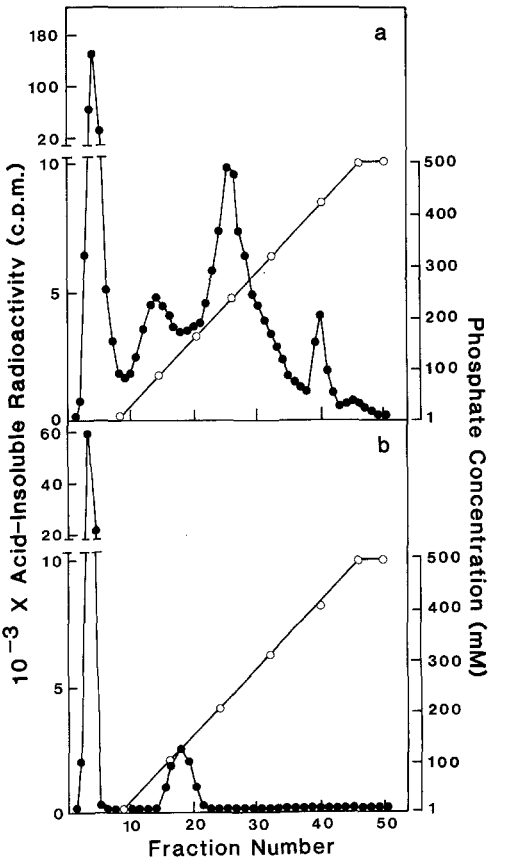

Figure 5. Hydroxylapatite column chromatography of ADP-ribose from permeabilized cells incubated with deoxyribonuclease I. The transferase reaction was as described for Fig. 4 except that DNAase was present at 100μg/ml. ADP-ribose was from cells in which degradation had proceeded for 0 min (a) or 60 min (b). In experiment (a) 322,809 c.p.m. were applied to the column and recovery was 105%. In experiment (b) 95,590 c.p.m. were applied and recovery was 99%.
Symbols as for Fig. 4.

ADP-ribose and its rate of removal in a 20 min period (Fig. 2). We infer from this that monomeric and polymeric ADP-ribose residues are removed at a common rate, at least in HeLa cells under our experimental conditions.

Our interpretation then of the biphasic $(ADP\text{-ribose})_n$ degradation kinetics, seen by others and ourselves, must be centred either on differential accessibility of various ADP-ribose residues [22] to degrading enzymes, or on the notion that the slowly turning over component has a different linkage to protein from the majority of residues and cleavage of these putative bonds is rate-limiting. For example, some monomeric ADP-ribose residues may be due to the non-enzymic attachment to proteins of moieties produced by glycohydrolase activity on polymeric ADP-ribose [23,1]. This could account for the observed build up and rate-limiting removal of mono(ADP-ribose) described by Wielckens and co-workers [4], if such a mechanism were operative in cell nuclei.

We note that the $T_{1/2}$ of our faster turning over component (12-15 min) is still considerably greater than values of 1 min or less previously reported [4-6]. This could be due to loss of degrading enzymes by leakage from the permeabilized cells.

Tanuma and colleagues [24] recently described two different glycohydrolases (nuclear and cytosolic) in HeLa S3 cells. Some of the ADP-ribose degradation we observed could then be due to entry of a cytosolic enzyme into the nuclei.

Berger's group [25] reported that 0.33mM NAD^+ inhibits glycohydrolase. However, they showed that the NAD concentration in permeabilized cells is very low and is essentially dictated by the experimenter. Therefore, the large difference in the rates of ADP-ribose degradation that we observe between ADPRT-stimulated cells and control cells cannot be due to a higher NAD^+ concentration in the control cells inhibiting degradation.

Why do cells require a mechanism for rapidly degrading $(ADP\text{-ribose})_n$? Sims *et al* [13] previously proposed a "suicide" model in which the global function of enhanced ADP-ribosylation, under conditions in which DNA is heavily damaged, is to deplete cellular NAD. The recycling of the

nicotinamide, produced in the transferase reaction, consumes phosphoribosyl pyrophosphate and ATP such that the cell cannot maintain ATP-dependent processes and consequently dies. The purpose of this scenario is to prevent aberrant DNA repair leading to the fixation of mutations, probably deleterious to multi-cellular organisms. A rapid degradation of $(ADP\text{-ribose})_n$ then would result from the elevated levels of ADP-ribose in the DNA damaged cells and this would ensure continued ADPRT activity, required to deplete cellular NAD. Without this rapid turnover all ADP-ribose acceptor sites might become quickly occupied, and existing chains maximally elongated. Further transferase activity and consequent NAD^+ depletion would then cease.

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