Activation of ADP-ribosyltransferase in polyamine-depleted mammalian cells

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Mammalian fibroblasts were cultured in the presence of α -methylornithine and/or methylglyoxal bis(guanyldrazone), which inhibit the synthesis of polyamines. This led to a decrease in the cellular content of the polyamines spermine and spermidine by up to 60% when the cells were grown in the presence of both drugs together. The activity of the chromatin-associated enzyme ADP-ribosyltransferase was enhanced 2–3-fold in the drug-treated cells when measured in cells subsequently rendered permeable to exogenous NAD^+ , the substrate for the transferase. This is a novel and surprising observation, since the transferase is invariably activated by the addition of polyamines to a suitable incubation system such as permeabilized cells, isolated nuclei or the purified enzyme. We found no evidence that the activation was due to the appearance of DNA strand breaks, by using a variety of procedures including both neutral [the 'nucleoid' technique of Cook & Brazell [(1975) *J. Cell Sci.* 19, 261–279; (1976) *J. Cell Sci.* 22, 287–302]] and alkaline sucrose-gradient centrifugation and gel electrophoresis, suggesting that this therefore may not be the only means of regulating the activity of ADP-ribosyltransferase and that polyamines may have a role to play in this regard *in vivo*.

Nuclei of eukaryotic cells contain an enzyme, ADPRT, that catalyses the cleavage of substrate NAD^+ with the concomitant covalent attachment of the ADP-ribose residue to chromosomal proteins. These may be modified with a single ADP-ribose moiety, mono(ADP-ribose), or with a homopolymer of repeating ADP-ribose units of various chain lengths, poly(ADP-ribose). Both histone and non-histone proteins become modified (Purnell *et al.*, 1980). The transferase is entirely dependent on DNA (Tsopanakis *et al.*, 1978) containing strand breaks for activity (Janakidevi & Koh, 1974; Miller, 1975; Halldorsson *et al.*, 1978; Berger *et al.*, 1978; Benjamin & Gill, 1980) and is activated whenever DNA breaks are induced (Whish *et al.*, 1975; Halldorsson *et al.*, 1978; Berger *et al.*, 1978, 1979; Juarez-Salinas *et al.*, 1979; Benjamin & Gill, 1980; Durkacz *et al.*, 1980, 1981a,b). These and other studies support proposals for a direct involvement of protein ADP-ribosylation in DNA repair (Shall, 1982; Creissen & Shall, 1982) and, perhaps an indirect one, in gene expression and cell

differentiation (Purnell *et al.*, 1980; Morioka *et al.*, 1980; Bredehorst *et al.*, 1981; Jackowski & Kun, 1981; Rickwood, 1982; Cherney *et al.*, 1982; Porteous & Pearson, 1982; Pearson, 1982; Althaus, 1982; Farzaneh *et al.*, 1982), events in which DNA strand breaks are thought to occur.

The polyamines, low-molecular-weight cations, have also been implicated as playing major roles in cell physiology (Tabor & Tabor, 1976; Jänne *et al.*, 1978; Heby, 1981). Reports in the literature have shown that the polyamines spermine and spermidine in general stimulate the activity of ADPRT when they are added to an incubation mixture containing isolated nuclei (Muller & Zahn, 1976; Tanigawa *et al.*, 1977, 1980; Perrella & Lea, 1978, 1979; Whitby *et al.*, 1979) or even to the purified transferase from rat liver nuclei (Kawamura *et al.*, 1981). The use of isolated nuclei has been criticized, however, since damage caused to the DNA during the isolation procedure leads to an artificial activation of the transferase. The use of whole cells rendered permeable to NAD^+ was considered to be more representative of the situation *in vivo* for studying ADP-ribosylation reactions, and we have since confirmed that polyamines added to this system also stimulate the transferase (Gordon *et al.*, 1982).

Here we present a different approach to study-

Abbreviations used: ADPRT, ADP-ribosyltransferase; DNAase, deoxyribonuclease; α MO, DL- α -methylornithine; MGBG, methylglyoxal bis(guanyldrazone) {1,1'-(methylethanediyldene)dinitrilo}diguanidine}.

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ing the putative involvement of polyamines in ADP-ribosylation reactions. We have diminished cellular polyamine content *in vivo*, by growing cells in the presence of inhibitors of polyamine biosynthesis, and have then examined the effect of this on the activity of ADPRT by using the permeabilized cell system. We report the surprising observation, in view of results described with systems purely *in vitro*, that a diminution of cell polyamine content leads to an activation of ADPRT and that this does not appear to result from DNA damage, suggesting the existence of additional means of controlling this enzyme activity *in vivo*.

Experimental

Materials

[6-³H]Thymidine (sp. radioactivity 20Ci/mmol) was purchased from Amersham International, Amersham, Bucks., U.K. Nicotinamide-[2,8-³H]-adenine dinucleotide (sp. radioactivity 20Ci/mmol) was prepared for us by Dr. W. J. Whish, University of Bath, U.K.

3-Aminobenzamide, dansyl (5-dimethylaminonaphthalene-1-sulphonyl) chloride, spermine tetrahydrochloride, spermidine trihydrochloride and pancreatic DNAase I were from Sigma Chemical Co., Poole, Dorset, U.K.; α MO was from Calbiochem-Behring, Bishops Cleeve, Herts., U.K.; MGBG was from Aldrich Chemical Co., Gillingham, Dorset, U.K.; hydroxyapatite was from Bio-Rad Laboratories, Watford, Herts., U.K.; proteinase K was from BDH Chemicals, Poole, Dorset, U.K.; and silica-gel 60 t.l.c. plates (without fluorescent indicator; 20cm $\times$ 20cm) were from E. Merck, Darmstadt, Germany. BHK-21/C13 cells and donor horse serum were from Flow Laboratories, Irvine, Ayrshire, Scotland, U.K.

Cell culture and harvesting

Baby-hamster kidney fibroblasts (BHK-21/C13) were grown routinely (Furneaux & Pearson, 1980) in monolayer culture at 37°C under CO₂/air (1:19) in Dulbecco's modification of Eagle's medium supplemented with 10% (w/v) horse serum. To diminish cell polyamine content, approx. 2×10^6 cells/Petri dish (9cm diam.) were seeded in medium containing dialysed horse serum and inhibitors of polyamine synthesis. These were α MO (5mM) and/or MGBG (10 μ M). Stock solutions of drugs (concentrated 100-fold) were prepared in 0.9% (w/v) NaCl, and 100 μ l of this was added to each Petri dish containing 10ml of medium; 100 μ l of 0.9% NaCl was added to control dishes. The cells were grown under these conditions for 24h and, after removal of medium,

washed twice in ice-cold phosphate-buffered saline (Adams, 1980) before harvest for polyamine and protein determinations.

Cells were harvested with trypsin/EDTA (1:4, v/v). Trypsin solution was 0.25% trypsin in Tris-buffered saline, consisting of 0.3% Trizma base, 0.8% NaCl, 0.02% KCl, 0.01% Na₂HPO₄ and 0.1% glucose, adjusted with HCl to pH7.7 at 25°C. EDTA solution was 0.5mM-EDTA in phosphate-buffered saline.

Determination of DNA synthesis, protein and polyamines

The incorporation of radioactivity from [³H]-thymidine into acid-insoluble material was used as a measure of accumulated DNA synthesis; cells were grown for 24h in the presence of [6-³H]thymidine (20Ci/mmol, 1 μ Ci/ml) before precipitation with 0.2M-HClO₄. Precipitates were dissolved in NaOH and the solution was acidified before counting for radioactivity. Protein concentrations were determined by the method of Lowry *et al.* (1951) and polyamines by the method of Dion & Herbst (1973). Putrescine was not measured, because the dansyl derivative migrated with α MO on the t.l.c. plates. Cells from two 9cm-diam. Petri dishes were harvested and pooled in phosphate-buffered saline. Then 0.5 μ l of 0.2M-HClO₄ was added to each cell sample, which was left on ice for 15min. Samples were homogenized with a motor-driven glass rod which fitted the tubes containing the solution, and the acid-insoluble material was removed by centrifugation at 8000g for 2min. Na₂CO₃·10H₂O (50 μ g/0.2ml of HClO₄ solution) was added to portions of the supernatant to raise the pH to about 10.3 for subsequent dansylation of the polyamines. Dansyl chloride (15mg/ml of acetone) was added to each sample, which was then incubated for 3h at 37°C. Excess dansyl chloride was removed by reaction with L-proline (100mg/ml) for 30min. Dansyl derivatives of the polyamines were extracted in toluene, which was subsequently evaporated in a stream of air. The derivatives were dissolved in 0.2ml of toluene, portions spotted on silica-gel G60 thin layers (without fluorescent indicator) and the plates developed in cyclohexane/ethyl acetate (3:2, v/v). The fluorescent dansyl-polyamines were detected under long-wavelength u.v. light, and appropriate spots scraped from the plates and extracted into conc.NH₃ (sp.gr. 0.880)/methanol (1:19, v/v). Fluorescence was determined at excitation 365nm and emission 535nm with a Perkin-Elmer 1000 spectrofluorimeter. Standard samples of spermine and spermidine were made to react with dansyl chloride in a similar manner and were used for quantification of the polyamines.

Cell permeabilization and ADPRT assays

The method of permeabilization was essentially that of Berger & Johnson (1976). Cells were pelleted (8000 g_{av} for 5 min) after washing and resuspended at 10×10^6 cells/ml in hypo-osmotic buffer (0.01 M-Tris/HCl, pH 7.8 at 4°C, containing 0.25 M-sucrose, 1 mM-EDTA, 30 mM-2-mercaptoethanol and 4 mM-MgCl₂). They were left at 0°C for 30 min and were then pelleted by brief bench centrifugation and resuspended at 10×10^6 cells/ml in hypo-osmotic buffer. ADPRT assays were performed in a final volume of 300 μ l containing 50 mM-Tris/HCl, pH 8.0 at 26°C, 10 mM-MgCl₂, 1 mM-dithiothreitol and 0.5 mM-[³H]NAD⁺ (28 μ Ci/mmol; 5 μ Ci/assay). Reactions were started by the addition of approx. 2×10^6 permeabilized cells and were incubated at 26°C for 30 min. Transferase activity was measured by the incorporation of radioactivity into trichloroacetic acid-precipitable material (Furneaux & Pearson, 1980).

ADP-ribose-degradation assays

Permeabilized cells were initially incubated for 30 min in the presence of [³H]NAD⁺ to label the protein-bound ADP-ribose residues. The conditions were as described above for ADPRT assays. After 30 min cells were pelleted and resuspended (2×10^6 cells) in 300 μ l of the transferase assay solution now containing 3-aminobenzamide at 5 mM. Incubations were continued for up to 90 min (still at pH 8.0 and 26°C). The loss of acid-insoluble radioactivity with time was taken as the measure of ADP-ribose degradation.

Control experiments showed that the transferase was completely inactivated under these conditions.

Hydroxyapatite-column chromatography

ADPRT assays were stopped after 30 min by adding to each tube 300 μ l of 40% (w/v) trichloroacetic acid solution containing 1% (w/v) nicotinamide, 10 mM-NAD⁺ and 2% (w/v) Na₄P₂O₇. Mixtures were left on ice for 60 min and tubes then centrifuged for 2 min at 8000 g (Eppendorf centrifuge 5412). Pellets were washed twice with 20% trichloroacetic acid and twice in 95% (v/v) ethanol. They were then dissolved in 50 μ l of 0.1 M-NaOH and incubated at 37°C for 60 min in order to release ADP-ribose chains from their covalent attachment to proteins. The solution was neutralized with 10 μ l of 0.5 M-HCl and incubated at 37°C for 60 min after addition of 60 μ l of 50 mM-Tris/HCl, pH 6.8 at 37°C, containing 20 mM-MgCl₂ and 50 μ g of pancreatic DNAase I. Then 30 μ l of water containing 50 μ g of proteinase K was added and the incubation continued at 37°C for another 60 min. Duplicate samples (assays) which had thus far been treated separately were now combined to give a total volume of 300 μ l; 250 μ l of this was applied to

a hydroxyapatite column (1 cm $\times$ 0.6 cm), which was eluted with a 25 ml step gradient of potassium phosphate buffer (1–500 mM; 50 steps, increasing by 10 mM at a time), and the fractions (0.5 ml) were counted for radioactivity (Farzaneh & Pearson, 1978).

Nucleoid sedimentation analysis

The nucleoid technique described by Cook & Brazell (1975, 1976) and Cook *et al.* (1976) was used, except that nucleoids were detected from the position of radioactive DNA in gradients instead of by absorbance measurements. Cells were grown for 24 h in the presence of [³H]thymidine at 1 μ Ci/ml to label the DNA. They were harvested with trypsin/EDTA, washed once in growth medium, once in Dulbecco's modified phosphate-buffered saline (this was a mixture of solutions A, B and C in the ratio 8:1:1, by vol.). Solution (A) was 0.03% KH₂PO₄, 0.14% Na₂HPO₄, 1% NaCl, 0.03% KCl at pH 7.2; (B) was 0.1% CaCl₂·H₂O, and (C) 0.2% MgCl₂·6H₂O and finally suspended in this solution at 10×10^6 cells/ml. Then 50 μ l of this cell suspension was layered, together with 100 μ l of freshly prepared lysis buffer (2 mM-Tris/HCl, pH 8.0 at 20°C, containing 2 M-NaCl, 100 mM-EDTA and 0.5% Triton X-100), on top of each gradient (5 ml) and left at room temperature for 20 min before centrifugation. Neutral linear sucrose gradients were used, consisting of 15–30% (w/v) sucrose in 10 mM-Tris/HCl, pH 8.0 at 20°C, containing 2 M-NaCl and 10 mM-EDTA. Gradients were centrifuged at 12000 rev./min (Fig. 5) or 10000 rev./min (Fig. 6) for 30 min at 20°C in a Beckman SW50.1 rotor. Six gradients were centrifuged simultaneously, with one or two tubes containing control cell lysates as a reference. Gradients were fractionated by pumping from the bottom either directly on to glass-fibre filters or into tubes (six drops/fraction), so that both radioactivity and refractive index could be measured. Filters were washed in 10% trichloroacetic acid, and ethanol, and were then dried and counted for radioactivity in FisoFluor 3 (Fisons, U.K.) at an efficiency of 30%.

Alkaline sucrose-density gradient centrifugation

Cells were grown for 24 h in small flasks (10 cm²) in the presence of [³H]thymidine (1 μ Ci/ml) to label the bulk DNA. After harvest and washing in phosphate-buffered saline, they were resuspended in 300–500 μ l of phosphate-buffered saline (about 1×10^6 – 2×10^6 cells). Depending on the number of cells and the acid-insoluble radioactivity content, samples of between 20–100 μ l were applied on top of linear gradients (4.6 ml) of 5–20% (w/v) sucrose in 0.3 M-NaOH, 10 mM-EDTA and 0.66 M-NaCl as described by Cheng & Nakayama (1982). Then

100 μ l of lysis buffer was added [this was the gradient solution, containing sucrose at 2.5% (w/v) and also 0.3% sodium *N*-lauroylsarcosine]. Centrifugation was carried out immediately at 20°C for 45 min at 25000 rev/min in a SW50.1 rotor in a Beckman L2-65B ultracentrifuge. Fractions (five drops) were collected from the bottom as described for the neutral gradients.

Results

Cell growth in the presence of α -MO and MGBG leads to a decrease in the cellular content of polyamines. α -MO inhibits ornithine decarboxylase (Mamont *et al.*, 1976), which leads to a decrease in putrescine and spermidine without influencing intracellular spermine concentration, because under these circumstances available putrescine and spermidine become rapidly converted into spermine (Wallace *et al.*, 1982). MGBG, on the other hand, inhibits *S*-adenosylmethionine decarboxylase (Fillingame & Morris, 1973), and both spermine and spermidine are decreased, whereas the concentration of putrescine rises, since it is no longer converted into spermidine. A decrease in all three of these polyamines can then be achieved by the combined use of these two drugs.

Table 1 shows that α -MO diminished total cellular polyamine content (measured as spermidine + spermine) by about 25%. MGBG did so by 37% and both drugs together did so by some 60%, compared with control cells grown in the absence of drugs. α -MO did not appreciably affect protein content or DNA synthesis during 24 h growth, but MGBG caused a 30% decrease in protein content (per plate) and a decrease in DNA synthesis of about 33%, measured by the accumulated incorporation of radioactive thymidine into acid-insoluble material. α -MO and MGBG to-

gether caused a 32% decrease in protein content per plate and in the extent of DNA synthesis.

Fig. 1 shows that in each case the diminution of cell polyamine content led to an enhancement of

Fig. 1. *Inhibitors of polyamine synthesis in vivo: effects on ADPRT activity subsequently measured in permeabilized cells*

Cells were grown for 24 h in the absence (control, ●) or presence of inhibitors of polyamine biosynthesis (○, α -MO; △, MGBG; ▲, α -MO and MGBG) as described for Table 1. They were then harvested, permeabilized and incubated in the presence of 0.5 mM-[3 H]NAD $^+$ to measure transferase activity. Results are means $\pm$ S.D. ($n = 3$). The S.D. is drawn on only one side of each experimental point for clarity.

Table 1. *Effect of α -methylornithine and methylglyoxal bis(guanyldrazide) on cell polyamine content and on DNA synthesis*
Cells were grown for 24 h in the absence (control) or presence of 5 mM- α -MO and/or 10 μ M-MGBG as described in the Experimental section. The results shown for polyamines and protein are the means ($\pm$ range) of two determinations. Although absolute values differed quite widely in a number of different experiments, the relative effects of the drugs were always as shown in the Table. Values for accumulated DNA synthesis during the 24 h are means $\pm$ S.D. ($n = 4$; different Petri dishes).

Addition	Concn. (mM)	Protein content (mg/plate)	$10^{-2} \times$ DNA synthesis (c.p.m./mg of protein)	Content (nmol/mg of protein)		
				Spermidine (spd)	Spermine (spm)	Total polyamines (spd + spm)
None	—	1.38 (1.30, 1.47)	21.76 ($\pm$ 1.59)	12.68 (11.64, 13.72)	8.52 (7.66, 9.38)	21.20
α -MO	5.0	1.51 (1.38, 1.64)	19.95 ($\pm$ 1.22)	7.14 (6.64, 7.61)	9.13 (6.10, 12.66)	16.27
MGBG	0.01	0.97 (1.03, 0.90)	13.78 ($\pm$ 0.56)	7.49 (6.82, 8.17)	5.81 (5.68, 6.07)	13.30
α -MO + MGBG	5.0, 0.01	0.95 (0.94, 0.95)	14.80 ($\pm$ 0.56)	6.32 (6.28, 6.36)	2.74 (2.02, 3.46)	9.06

ADPRT activity when this was measured in drug-treated cells subsequently rendered permeable to exogenous NAD^+ . The greatest stimulatory effect, some 3-fold, was seen in cells grown in the presence of MGBG alone, slightly greater, in fact, than in cells treated with αMO and MGBG together. Control experiments showed that the drugs added directly to permeabilized cells had no stimulatory effect on the transferase.

This increase in ADP-ribose synthesis did not result from a decrease in $(\text{ADP-ribose})_n$ degradation. Experiments to measure degradation were carried out by first incubating permeabilized cells with $[^3\text{H}]\text{NAD}^+$ for 30 min at 26°C to synthesize ADP-ribose. Loss of acid-insoluble radioactivity in the presence of 5 mM-3-aminobenzamide was then measured over the next 90 min, also at 26°C . This varied from being barely detectable up to a maximum of about 30%. No significant difference was observed between the situation in control and drug-treated cells (Fig. 2). The increase in ADP-ribose synthesized in the drug-treated cells was, by contrast, some 200–300%.

It is to be expected that any increase in ADP-ribose synthesis will be reflected either in a greater chain length of $(\text{ADP-ribose})_n$ residues attached to protein and/or an increase in their total number. Hydroxyapatite column chromatography (Tanaka *et al.*, 1977) has been used routinely to measure this.

Fig. 2. Inhibitors of polyamine synthesis *in vivo*: effects on the degradation of protein-bound ADP-ribose subsequently measured in permeabilized cells

Cells were grown as described for Fig. 1. They were harvested after 24 h, permeabilized and incubated with 0.5 mM- $[^3\text{H}]\text{NAD}^+$ for 30 min to label ADP-ribose. After this time they were placed in medium containing 5 mM-3-aminobenzamide and the incubation was continued. The Figure shows the loss of acid-insoluble radioactivity with time. ●, Control (100% = 332 c.p.m.); ○, cells grown with αMO and MGBG (100% = 684 c.p.m.). Results are means $\pm$ S.D. ($n = 3$).

Chromatographic analysis of $(\text{ADP-ribose})_n$ residues cleaved from protein with alkali showed that a wide spectrum of chain lengths had been synthesized (Fig. 3). The first major peak of radioactivity (fractions 1–4) represents mainly mono- $(\text{ADP-ribose})_n$, as shown by polyethyleneimine-cellulose t.l.c., and presumably represents new chain synthesis in these permeabilized cells. There is no obvious difference in the overall spectrum of chain lengths synthesized in the cells grown under the different conditions. It is not clear whether this is because the enhanced ADPRT activity in the polyamine-depleted cells leads to an overall increase in synthesis of all $(\text{ADP-ribose})_n$ chains, or that the similar profiles reflect the limitations on the sensitivity of this technique to detect relatively small changes in total chain-length distribution spread perhaps over a broad size range. Any apparent differences in the profiles shown (Figs. 3a–3d) are within the variations given by an individual sample.

In contrast with this, we could clearly detect a different elution profile for ADP-ribose residues obtained from permeabilized cells incubated with pancreatic DNAase I to activate the ADPRT by creating DNA strand breaks (Fig. 4). This shows a greater proportion of radioactivity in the longer ADP-ribose chains (from fraction 21). The extent of ADPRT activation after this DNAase treatment, however, was about 8-fold, some 2–3-fold greater than that in the polyamine-depleted cells (Fig. 1).

We have not given further detailed consideration in this present study to the question of chain lengths, such as that carried out by Tanigawa *et al.* (1980), but instead have addressed ourselves mainly to the question of what could have caused the increase in ADP-ribose synthesis in the drug-treated cells. In particular, we have considered whether the drug treatments could have resulted in DNA damage, now firmly established as a cause of ADPRT activation. For this we used the neutral-sucrose-gradient technique described by Cook & Brazell (1976) and Cook *et al.* (1976). In this technique cells are lysed in non-ionic detergent directly on top of the gradient. During centrifugation in the presence of appropriate salt concentration the DNA sediments as a structure referred to as a 'nucleoid', which is basically considered to be a nucleus devoid of most of its protein. Sedimentation analysis shows that the DNA in these nucleoid structures behaves as a compact molecule containing individual domains of supercoiled DNA. Any damage to the DNA, in the form of phosphodiester-bond breaks, causes the DNA in the region of the break to relax, thus forming a more open structure. This is reflected in a slower rate of sedimentation in the gradients. The

Fig. 3. Hydroxyapatite column chromatography of ADP-ribose chains from polyamine-depleted cells. Cell growth and permeabilization conditions were as described for Fig. 1. Permeabilized cells were incubated with [^3H]NAD $^+$ for 30 min. The radiolabelled ADP-ribose chains were then cleaved from protein and chromatographed on the hydroxapatite columns. (a) ADP-ribose chains from control cells: 23 000 c.p.m. was applied to the column, and the recovery of radioactivity was 96%. (b) Cells grown for 24 h in the presence of 5 mM αMO ; 23 790 c.p.m. was applied, and the recovery was 106%. (c) Cells grown with 10 μM -MGBG; 23 790 c.p.m. was applied, and recovery was 124%. (d) Cells grown with both αMO and MGBG; 29 450 c.p.m. was applied, and recovery was 107%. Δ , potassium phosphate concentration.

sedimentation behaviour of the nucleoids can be quantitatively related to the extent of DNA damage, within certain limits, and can be expressed as the ratio of the distance sedimented by the damaged nucleoids to that of the control undamaged nucleoids (Cook & Brazell, 1975; Durkacz *et al.*, 1981b).

Fig. 5(b) shows that the nucleoids from cells grown in the presence of αMO and MGBG sedimented at the same rate as the control nucleoids (Fig. 5a), indicating that the drug-treated cells did not contain a greater number of DNA strand breaks. A similar result was also obtained when cells were grown in the presence singly of either αMO or MGBG. We have performed several (six) gradient analyses (under various conditions of time and rotor speed) and have not observed any significant difference in sedimentation rate between nucleoids from control and drug-treated cells. The position of nucleoids from γ -irradiated

cells is shown (Fig. 5a, arrow) for reference to be clearly different from control cell nucleoids. The 500rd used would be expected to produce about 2000–2400 single-strand breaks per DNA genome (Durkacz *et al.*, 1981b). The limit of this nucleoid technique is reached when the DNA contains fewer than about 200–300 single-strand breaks.

We next considered the possibility that putative damage to the DNA in the drug-treated cells was being rapidly repaired, thereby remaining undetected in our experiments. Cells were grown in the presence of 3-aminobenzamide, a potent inhibitor of the ADPRT whose activity is essential for the ligation of DNA-strand breaks (Creissen & Shall, 1982). Nucleoids from such cells sediment more slowly than those from control cells (Fig. 5c), evidence of accumulated DNA damage. However, nucleoids from cells grown in the combined presence of 3-aminobenzamide and the inhibitors of polyamine synthesis exhibited identical sedi-

Fig. 4. Hydroxyapatite column chromatography of ADP-ribose chains from permeabilized cells incubated with deoxyribonuclease

Exponentially growing cells were permeabilized for 30 min and then incubated for 30 min in the presence of [^3H]NAD $^+$ under conditions described for Fig. 1. Pancreatic DNAase I (final concn. 20 $\mu\text{g}/\text{ml}$) was added at the start of the ADPRT assay. All subsequent procedures are as described in Fig. 3 legend. ADP-ribose chains were from either (●) control cells (30394 c.p.m. applied to the column; recovery 86%) or (○) cells incubated with DNAase (45612 c.p.m. applied; recovery 95%).

mentation characteristics (Fig. 5d). Therefore, although DNA strand breaks also accumulated in these cells, there was no additional contribution caused by the presence of the αMO and MGBG, supporting the conclusions drawn from results shown in Figs. 5(a) and 5(b). The presence of 3-aminobenzamide in the growth medium together with αMO and MGBG did not influence the effects of latter drugs in decreasing the cell polyamine content.

We do not attribute significance to the one or two fractions' difference in sedimentation positions of nucleoids (cf. Fig. 5a with Fig. 5b, and Fig. 5c with Fig. 5d), since the precise position of any individual sample in different tubes varied over three fractions.

To be certain that perhaps different extents of DNA damage were not occurring after cell growth under the conditions used, we carried out nucleoid sedimentation analysis on cells that had been permeabilized for 30 min, and on cells that were permeabilized and then incubated for a further 30 min under conditions for the ADPRT assay (Fig. 6). After the 30 min permeabilization, the nucleoids from the drug-treated cells did not sediment as fast as those from control cells (Figs. 6e and 6b), although these latter seemed to be undergoing some transition. However, after the full 60 min

period *in vitro*, nucleoids from both the control and the drug-treated cells migrated to the same position [ratios were 0.73 (Fig. 6c, control) and 0.62 (Fig. 6f, drug-treated)]. We do not consider these to be significantly different from each other, and, if the work of Durkacz *et al.* (1981b) can be extrapolated to our studies, these ratios represent only up to 600–700 DNA strand breaks per genome. In a duplicate experiment the ratios after the complete 60 min incubation (the 30 min permeabilization time only was omitted from this experiment) were 0.95 (control) and 0.88 (drug-treated); again we do not consider these to be significantly different from each other. These ratios are sufficiently close to 1.0 (control cells not incubated for 60 min *in vitro*) that it is uncertain whether any DNA strand breaks are present at all. If they are present, however, their maximum number is about 300 breaks per genome (Durkacz *et al.*, 1981b).

The nucleoid technique is considered to be more sensitive than alkaline-sucrose-gradient analysis for detecting DNA strand breaks. However, it was demonstrated that some DNA breaks cannot be detected by the nucleoid technique, because they are masked by proteins (possibly repair enzymes) that cannot be removed by the high salt concentrations of the neutral sucrose gradients, although they are detectable by using alkaline sucrose gradients (Charles & Cleaver, 1982). Using these gradients, however, we still did not detect any difference between the sedimentation characteristics of DNA from control and drug-treated cells (Fig. 7; neither the presence of material near the top of the gradient in Fig. 7b nor the difference in peak widths represents reproducible differences); neither did we when analysing the DNA by neutral and alkaline agarose-gel electrophoresis (results not shown).

Discussion

Our observation that an enhancement of ADPRT activity resulted from decreasing cellular polyamine content is a novel one, in view of the numerous reported stimulatory effects on the transferase caused by the presence of polyamines *in vitro*. It is unclear why the extent of enzyme activation (see Fig. 1) appears greater in cells grown in the presence of just one drug, MGBG, than when they were grown with both MGBG and αMO , since the depletion of cellular polyamine content was greater in this latter case (Table 1). We can only conclude that there is probably not a direct relationship between the precise cellular polyamine content and the degree to which the transferase is activated.

Other reports which concur with our findings are (1) the observation that the intrinsic activity of

Fig. 5. Nucleoid sedimentation analysis of drug-treated cells

All cells were grown in the presence of [^3H]thymidine at $1\ \mu\text{Ci/ml}$ to label the bulk DNA. Nucleoids were from (a) control cells, (b) cells grown for 24 h in the combined presence of αMO (5 mM) and MGBG ($10\ \mu\text{M}$), (c) cells grown for 24 h in the presence of 3-aminobenzamide (5 mM), or (d) cells grown for 24 h in the combined presence of 3-aminobenzamide (5 mM), αMO (5 mM) and MGBG ($10\ \mu\text{M}$). The position of nucleoids from cells γ -irradiated with 500 rd is shown by the arrow in (a); Δ indicates refractive index. These gradients were centrifuged at 12000 rev./min for 30 min; direction of sedimentation is from right to left.

ADPRT, as measured in permeabilized Ehrlich ascites-tumour cells, exhibited an inverse correlation with ornithine decarboxylase activity (Bredehorst *et al.*, 1979) and (2) work from Frank Perrella's laboratory (Perrella, 1982) showing that polyamines added to permeabilized lymphocytes after mitogen stimulation only enhance ADPRT activity when these cells are synthesizing DNA, some 30–48 h after mitogen stimulation. At earlier times (0–6 h), before the initiation of DNA synthesis, polyamines inhibited ADPRT activity, leading Perrella (1982) to suggest that they might modulate the activity of the transferase *in vivo*.

On the basis of these studies we can offer testable explanations for our results. We have used

exponentially growing cells, a population thus containing cells in different phases of the growth cycle. If Perrella's (1982) findings are applicable to tissue-culture cells in cycle, we can predict that ADPRT may be activated by endogenous polyamines in the cells that are in S-phase and inhibited in those that are not, particularly those in G1-phase. The control transferase activity that we measure might therefore be an average value. Since inhibitors of polyamine synthesis, particularly MGBG (Heby *et al.*, 1977; Rupniak & Paul, 1978), are known to block cells at the G1/S-phase border, this would dramatically increase the proportion of cells whose ADPRT would normally be inhibited by endogenous polyamines. However,

Fig. 6. Nucleoid sedimentation analysis of permeabilized cells

The cellular DNA was in all cases labelled with [3 H]thymidine as in Fig. 5. Nucleoids were from (a) control cells, (b) control cells that were permeabilized for 30 min before being loaded on gradients, (c) control cells that were permeabilized for 30 min and then incubated for a further 30 min at 26°C in the ADPRT assay mixture (this contained non-radioactive NAD $^+$ only, at 0.5 mM), (d) cells grown for 24 h with 5 mM- α MO and 10 μ M-MGBG, (e) cells grown with the drugs and then permeabilized for 30 min before centrifugation, and (f) cells grown with the drugs and subsequently permeabilized for 30 min and incubated for another 30 min in the ADPRT mixture. Gradients were centrifuged at 10000 rev./min for 30 min; direction of sedimentation is from right to left.

Fig. 7. Alkaline sucrose-density-gradient centrifugation. Cells were incubated with [^3H]thymidine as in Fig. 5 and were grown for 24h either in the absence (control, *a*) or presence of 5mM- αMO and 10 μM -MGBG (*b*). Samples were lysed on top of alkaline gradients; direction of sedimentation is from right to left.

since such drugs decrease the cell polyamine content (Table 1), this would relieve the putative inhibitory effect of these polyamines. It is consequently feasible that what we have described as an apparent activation of ADPRT activity in the polyamine-depleted cells is in fact a relief from inhibition by endogenous polyamine. This enhanced transferase activity therefore would not be associated with DNA strand breaks.

We wish to emphasize at this point that our results cannot rule out the presence of a small number of DNA strand breaks in the polyamine-depleted cells, since the lower limit of the most sensitive of the techniques that we used to detect these (the nucleoid method) is about 300 single-

strand breaks per genome. Clearly our nucleoids have possibly accumulated up to 600 or 700 DNA strand breaks during the 60 min incubation period *in vitro* (see the Results section and Figs. 6c and 6f). Results after only the 30 min permeabilization (compare Figs. 6b and 6e) suggest that the rate of accumulation of breaks may be different in the control and drug-treated cells. However, the radio-labelled NAD^+ is present only during the second half of the total 60 min incubation period *in vitro*. It would therefore be necessary to assume that the drug-treated cells contained up to 600 or 700 DNA breaks per genome more than the control cells for a sustained time during the 30 min for which the [^3H]NAD $^+$ was present, in order for this low number of DNA strand breaks to be responsible for the enhanced transferase activity that we describe.

We think this is unlikely for two main reasons. (1) Skidmore *et al.* (1979) showed that doses of γ -irradiation sufficient to create about 1000 DNA strand breaks (or less) per genome (about 250rd) do not cause a measurable increase in ADPRT activity in permeabilized cells. In fact, after 15000rd the transferase was activated only about 4-fold. (2) We observed that when ADPRT was activated to an extent comparable with that in the polyamine-depleted cells, by incubating permeabilized cells with an appropriate low concentration of deoxyribonuclease (10 μg /assay), the DNA was heavily damaged and was readily detected as such on alkaline sucrose gradients, under conditions in which the nuclease was rendered inactive.

Further work is required to consider our proposals, since they impinge on the very important question of whether the occurrence of DNA strand breaks is in fact the only means of activating the ADPR transferase.

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