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Poly(ADP-ribose) Polymerase Inhibitors Preserve Nicotinamide Adenine Dinucleotide and Adenosine 5′-Triphosphate Pools in DNA-Damaged Cells: Mechanism of Stimulation of Unscheduled DNA Synthesis†

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ABSTRACT: Inhibitors of poly(ADP-ribose) polymerase stimulated the level of DNA, RNA, and protein synthesis in DNA-damaged L1210 cells but had negligible effects in undamaged L1210 cells. The poly(ADP-ribose) polymerase inhibitors stimulated DNA repair synthesis after cells were exposed to high concentrations of *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (68 and 136 μM) but not after exposure to low concentrations (13.6 and 34 μM). When the L1210 cells were exposed to 136 μM *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine, the activation of poly(ADP-ribose) polymerase resulted in the rapid depletion of oxidized nicotinamide adenine dinucleotide (NAD^+) levels and subsequent depletion of adenosine 5′-triphosphate (ATP) pools. After low doses of *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (13.6 μM), there were only small decreases in NAD^+ and ATP. Poly(ADP-ribose) polymerase inhibitors prevented the rapid fall in NAD^+ and ATP pools. This preservation of the ATP pool has a permissive effect on energy-dependent functions and accounts for the apparent stimulation of DNA, RNA, and protein synthesis. Thus, the mechanism by which poly(ADP-ribose) polymerase inhibitors stimulate DNA, RNA, and protein synthesis in DNA-damaged cells appears to be mediated by their ability to prevent the drastic depletion of NAD^+ pools that occurs in heavily damaged cells, thereby preserving the cells' ability to generate ATP and maintain energy-dependent processes.

Poly(ADP-ribose)[1] polymerase, a chromatin-bound enzyme, cleaves NAD^+ to yield nicotinamide and ADP-ribose, and then the same enzyme polymerizes successive ADP-ribose residues to synthesize poly(ADP-ribose) (Hayaishi & Ueda, 1977). While the function(s) of poly(ADP-ribose) is (are) not yet clearly established, a number of studies have shown that its synthesis is stimulated by DNA-damaging agents such as MNNG, Me_2SO_4, MNU, and UV, γ, or X irradiation (Berger et al., 1979, 1980; Jacobson, M. K., et al., 1980; Durkacz et al., 1980; Sudhakar et al., 1979; Skidmore et al., 1979; Benjamin & Gill, 1980). It has also been shown that the increase in poly(ADP-ribose) polymerase activity stimulated by DNA damage is associated with a decrease in cellular NAD^+ levels and an increase in intracellular poly(ADP-ribose) levels (Jacobson, M. K., et al., 1980; Durkacz et al., 1980; Skidmore et al., 1979; Jaurez-Salinas et al., 1979; Sims et al., 1982). The possiblity that poly(ADP-ribose) synthesis is required during the DNA repair process is suggested by the observations that NAD^+-starved 3T3 cells loose their ability to repair MNNG-induced DNA damage (Jacobson, E. L., et al., 1980), that poly(ADP-ribose) polymerase inhibitors retard the rejoining of DNA strand breaks in Me_2SO_4-treated L1210 cells (Durkacz et al., 1980), and that the cytotoxicity of some DNA-damaging agents is enhanced by poly(ADP-ribose) polymerase inhibitors (Durkacz et al., 1980).

In contrast to some of the studies outlined above, we have recently shown that the unscheduled DNA synthesis that occurs in normal human lymphocytes after treatment with DNA-damaging agents such as UV irradiation, Me_2SO_4, or MNNG can be stimulated by the addition of nicotinamide analogues to the culture medium (Berger & Sikorski, 1980). Miwa et al. showed that similar stimulation of unscheduled DNA synthesis occurred when DNA-damaged lymphocytes were treated with a series of nicotinamide analogues (Miwa et al., 1981), and we showed that this stimulation was dependent on the concentration of the agents in the culture medium (Berger & Sikorski, 1980) and on their potency as inhibitors of poly(ADP-ribose) polymerase (Sims et al., 1982). Stimulation of DNA repair synthesis by poly(ADP-ribose) polymerase inhibitors has also been observed in DNA-damaged rat hepatocytes (Althaus et al., 1980). Since lymphocytes and hepatocytes are resting intermitotic cells with negligible levels of replicative DNA synthesis, it seemed possible that the ability of poly(ADP-ribose) polymerase inhibitors to stimulate un-

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[1] Abbreviations: ADP-ribose, adenosine 5′-diphosphate ribose; dThd, thymidine; dTMP, thymidine 5′-phosphate; Leu, leucine; MNNG, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine; MNU, *N*-methyl-*N*-nitrosourea; NAD^+, nicotinamide adenine dinucleotide (oxidized); UMP, uridine 5′-phosphate; Urd, uridine; εRAdo, 1,N^6-etheno-2′-ribosyladenosine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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Table I: Effect of Nicotinamide on DNA Synthesis in Undamaged and MNNG-Treated L1210 Cells[a]

	DNA synthesis (dpm [^{3}H]dTMP/10^6 cells in 6 h)					
	expt 1		expt 2		expt 3	
treatment	−HU	+HU	−HU	+HU	−HU	+HU
control (undamaged)	1 690 000	22 000	1 448 000	36 000	1 630 000	35 000
control + nicotinamide	1 570 000	23 000	1 376 000	38 000	1 522 000	41 000
136 μM MNNG	69 000	20 800	65 000	19 000	82 000	15 000
136 μM MNNG + nicotinamide	92 000	40 600	175 000	93 000	213 000	87 000

[a] Cell cultures were pretreated for 15 minutes with 10 mM hydroxyurea (+HU) or with an equivalent volume of saline (−HU). At time zero, cells were treated with MNNG in Me_2SO or an equivalent volume of Me_2SO (controls). As indicated, cultures were adjusted to contain 2 mM nicotinamide or an equivalent volume of saline. [^{3}H]dThd was added to label DNA. After a 6-h incubation in the presence or absence absence of 10 mM hydroxyurea, the incorporation of [^{3}H]dTMP into DNA was measured as described under Methods.

scheduled DNA synthesis might be confined to nonreplicating cells. In the present study, we used rapidly growing L1210 cells to determine whether poly(ADP-ribose) polymerase inhibitors could stimulate DNA repair synthesis in replicating cells and to investigate the mechanisms involved in this stimulation.

Experimental Procedures

Materials

Nicotinamide and hydroxyurea were purchased from Sigma Chemical Co. (St. Louis, MO). Benzamide was obtained from Aldrich (Milwaukee, WI). [*methyl*-^{3}H]Thymidine (sp act. 73–80 Ci/mmol) and [5,6-$^3H_2$]uridine (sp act. 37.6 Ci/mmol) were purchased from New England Nuclear (Boston, MA). [4,5-$^3H_2$]Leucine (sp act. 72 Ci/mmol) was purchased from Amersham (Arlington Heights, IL). A 4 mm i.d. × 300 mm MCH-10 reversed-phase column for high-pressure liquid chromatography (HPLC) was purchased from Varian Associates (Sunnyvale, CA).

Methods

Cells and Culture Conditions. Cells were grown at 37 °C in α-modified Eagle's medium buffered with 25 mM Hepes, pH 7.2, and supplemented with 10% fetal calf serum, 50 units/mL penicillin, and 50 μg/mL streptomycin. The medium contained 8 μM nicotinamide. Some cultures were adjusted to contain 10 mM hydroxyurea 15 min prior to the addition of MNNG and/or Me_2SO. Logarithmically growing L1210 cells [(0.3–1.0) × 10^6/mL] (doubling time 18 h) were treated with MNNG that was freshly dissolved in Me_2SO, or in the case of controls, an equivalent volume of Me_2SO was added to the cultures to give a final concentration of 0.2%.

Measurement of DNA, RNA, and Protein Synthesis. To measure DNA synthesis, MNNG- and/or Me_2SO-treated cells were immediately distributed into sterile plastic culture tubes and [^{3}H]dThd was added to a final concentration of 0.5 μCi/mL (sp act. 80 Ci/mmol). Incorporation of [^{3}H]dTMP into trichloroacetic acid insoluble material was measured after a 6-h incubation at 37 °C as previously described (Sims et al., 1982). Six hours was selected as the optimal incubation period to see maximal stimulation of unscheduled DNA synthesis (Sims et al., 1982). Radioactivity incorporated under these conditions was alkali stable and sensitive to DNase I, indicating that DNA was being specificly labeled.

RNA and protein synthesis were measured by incubating the cells with 0.05 μCi/mL [^{3}H]Urd (sp act. 37.6 Ci/mmol) or with 5 μCi/mL [^{3}H]Leu (sp act. 72 Ci/mmol). Incorporation of [^{3}H]UMP into 10% trichloroacetic acid insoluble material and incorporation of [^{3}H]Leu into 20% trichloroacetic acid insoluble material were performed as previously described for DNA labeling (Sims et al., 1982). Label incorporated in [^{3}H]Urd-treated cells was alkali labile but cold acid stable, indicating that RNA was being specificly labeled.

Determination of NAD^+ and ATP Levels. Duplicate samples of cell suspension were removed at the indicated times, the cells were collected by centrifugation, and NAD^+ and ATP were extracted with perchloric acid as previously described (Sims et al., 1982). Each sample was then assayed for NAD^+ and ATP in duplicate by enzymatic cycling techniques (Kato et al., 1973).

Measurement of Poly(ADP-ribose) Levels. Cells were collected by centrifugation, the medium was aspirated, and cells were precipitated by the addition of ice-cold 20% trichloroacetic acid. Cell pellets were washed twice with 20% trichloroacetic acid, and the insoluble material was dissolved in 6 M guanidine containing 0.1 M potassium phosphate buffer, pH 8.6. Poly(ADP-ribose) was selectively adsorbed to a dihydroxyboryl-Sepharose column and then degraded to nucleosides by treatment with snake venom phosphodiesterase and bacterial alkaline phosphatase (Sims et al., 1980, 1982). The nucleosides were eluted and then incubated with chloracetaldehyde to convert adenine ring compounds to their highly fluorescent 1,N^6-etheno derivatives. 1,N^6-Etheno-2′-ribosyladenosine (εRAdo), which is specifically derived from poly(ADP-ribose), was separated from other fluorescent compounds by reversed-phase high-pressure liquid chromatography and quantified fluorometrically to measure cellular levels of poly(ADP-ribose) (Sims et al., 1980).

Results

Effect of Nicotinamide on DNA Synthesis in Control and MNNG-Treated L1210 Cells. Table I shows the effect of nicotinamide on DNA synthesis in L1210 cells measured in the presence and absence of hydroxyurea. In the absence of hydroxyurea, the L1210 cells showed a high level of [^{3}H]-dTMP incorporation, reflecting the high level of DNA synthesis in these logarithmically growing cells. Addition of 10 mM hydroxyurea to the culture medium suppressed replicative DNA synthesis by 97%. For example, in experiment 1, addition of 10 mM hydroxyurea to the replicating cells suppressed [^{3}H]dTMP incorporation from 1 690 000 dpm/10^6 cells in 6 h to 22 000 dpm/10^6 cells in 6 h. The addition of 2 mM nicotinamide to control cells had a negligible effect on replicative DNA synthesis and did not alter the ability of hydroxyurea to suppress this DNA synthesis. For example, in experiment 1, control cells treated with hydroxyurea incorporated 22 000 dpm/10^6 cells in 6 h and cells treated with hydroxyurea and nicotinamide incorporated 23 000 dpm/10^6 cells in 6 h.

When L1210 cells were treated with the DNA-damaging agent MNNG at a concentration of 136 μM, there was a 95% reduction in the rate of [^{3}H]dTMP incorporation compared to that in undamaged controls. For example, in experiment 1, MNNG treatment caused [^{3}H]dTMP incorporation to decrease from 1 690 000 to 69 000 dpm/10^6 cells in 6 h. In-

cubation of MNNG-treated cells in 10 mM hydroxyurea resulted in an even lower level of [^{3}H]dTMP incorporation than that which occurred in cells treated with MNNG alone. For example, in experiment 1, cells treated with MNNG alone incorporated 69 000 dpm/10^6 cells in 6 h while those treated with MNNG plus hydroxyurea incorporated 20 800 dpm/10^6 cells in 6 h. The level of incorporation in the cells treated with MNNG alone presumably reflects a combination of the residual replicative DNA synthesis and the MNNG-induced DNA repair synthesis. Since the major effect of hydroxyurea is to suppress the replicative component of DNA synthesis (Cleaver, 1969; Brant et al., 1972), the 20 800 dpm incorporated in the presence of hydroxyurea presumably reflects the level of DNA repair synthesis in the MNNG-treated cells. It is also possible that 10 mM hydroxyurea has some suppressive effect on DNA repair synthesis (Collins et al., 1977; Cornelius, 1978; Francis et al., 1979); however, as shown below, this does not alter the detection of the nicotinamide effect. Thus, incubation of MNNG-treated cells with 2 mM nicotinamide resulted in an increase in the level of DNA synthesis, and this occurred whether the cells were incubated in the presence or absence of hydroxyurea. For example, in experiment 1, when MNNG-treated cells were incubated with nicotinamide, the level of DNA synthesis increased from 69 000 to 92 000 dpm/10^6 cells in 6 h. When the same study was conducted in the presence of hydroxyurea, the addition of nicotinamide to the MNNG-treated cells caused the level of DNA synthesis to increase from 20 800 to 40 600 dpm/10^6 cells in 6 h.

The second and third experiments in Table I confirm that 2 mM nicotinamide had no significant effect on the rate of [^{3}H]dTMP incorporation in undamaged cells. In contrast, nicotinamide stimulated [^{3}H]dTMP incorporation in MNNG-treated L1210 cells, and this stimulation occurred whether the cells were incubated in the presence or absence of hydroxyurea. Thus, the ability of nicotinamide to stimulate [^{3}H]dTMP incorporation was specific for DNA-damaged cells, and it occurred in the presence and absence of hydroxyurea. The magnitude of the stimulation varied from experiment to experiment within the range of 1.3–5.8-fold with an average stimulation of about 2–3-fold. Thus the replicating L1210 cells were similar to resting intermitotic cells in that nicotinamide stimulated the level of DNA synthesis in DNA-damaged cells but not in control, undamaged cells.

Effect of Nicotinamide and Benzamide on DNA, RNA, and Protein Synthesis. To further evaluate the effect of the poly(ADP-ribose) polymerase inhibitors in the response to DNA damage, we measured the effect of nicotinamide and benzamide on the level of DNA, RNA, and protein synthesis in MNNG-treated cells. Table II shows that both nicotinamide and benzamide stimulated all three of these synthetic processes. When MNNG-treated cells were labeled with [^{3}H]dThd to assess DNA synthesis, the cells incorporated 18 300 dpm/10^6 cells in 6 h. In the presence of nicotinamide, [^{3}H]dTMP incorporation increased 4.2-fold, and in the presence of 2 mM benzamide, incorporation increased 6-fold. This greater stimulation by benzamide relative to nicotinamide is in agreement with our previous study showing that the degree of stimulation is dependent on the potency of the analogue as an inhibitor of poly(ADP-ribose) polymerase (Sims et al., 1982). When the MNNG-treated cells were labeled with [^{3}H]Urd to assess RNA synthesis, the cells incorporated 3500 dpm/10^6 cells in 6 h. In the presence of nicotinamide, there was a 3.8-fold stimulation of [^{3}H]UMP incorporation, and in the presence of benzamide, there was a 5.3-fold increase in [^{3}H]UMP incorporation. Similarly,

Table II: Stimulation of DNA, RNA, and Protein Synthesis by Poly(ADP-ribose) Polymerase Inhibitors[a]

treatment	macromolecular synthesis (dpm/10^6 cells in 6 h)		
	[^{3}H]dTMP	[^{3}H]UMP	[^{3}H]Leu
136 μM MNNG	18 300	3 500	5 100
136 μM MNNG + nicotinamide	77 300	13 300	20 900
136 μM MNNG + benzamide	109 000	18 700	30 600

[a] Cultures were pretreated for 15 min with 10 mM hydroxyurea. At time zero, all cells were treated with MNNG, and where indicated, 2 mM nicotinamide or benzamide was added. Cultures were labeled to assess DNA, RNA, and protein synthesis by the addition of [^{3}H]dThd, [^{3}H]Urd, or [^{3}H]Leu. After a 6-h incubation in hydroxyurea-containing medium, incorporation of label was measured as described under Methods.

when MNNG-treated cells were labeled with [^{3}H]Leu to assess protein synthesis, the cells incorporated 5100 dpm/10^6 cells in 6 h. In the presence of nicotinamide, there was a 4-fold stimulation of leucine incorporation, and in the presence of benzamide, there was a 6-fold stimulation in leucine incorporation. Analogous experiments to those conducted above showed that 2 mM nicotinamide and benzamide had no effect on the level of RNA and protein synthesis in undamaged cells. While this paper was in preparation, Taniguchi et al. (1982) reported that poly(ADP-ribose) polymerase inhibitors stimulated RNA synthesis in DNA-damaged cells. They found that the synthesis of both mRNA and rRNA was increased under these conditions. Their observations in combination with our findings on DNA, RNA, and protein synthesis show that poly(ADP-ribose) polymerase inhibitors have broad effects on cellular synthetic processes in DNA-damaged cells.

Since massive DNA damage can stimulate poly(ADP-ribose) synthesis with subsequent depletion of cellular NAD^+ pools (Jacobson, M. K., et al., 1980; Durkacz et al., 1980; Goodwin et al., 1978), it seemed possible that inhibitors of the polymerase might prevent this depletion and thus, by preserving the NAD^+ pool, contribute to the broad metabolic effects observed in the presence of the nicotinamide analogues. This seemed especially likely when it is considered that NAD^+ is required for ATP synthesis (Ernster, 1977) and that depletion of NAD^+ pools could also lead to a fall in ATP levels. In fact, it has already been shown that treatment of cells with γ irradiation and with neocarzinostatin leads to NAD^+ depletion and an associated fall in ATP levels (Goodwin et al., 1978). ATP depletion could result in inhibition of energy-requiring processes such as DNA, RNA, and protein synthesis. Thus, the addition of poly(ADP-ribose) polymerase inhibitors to DNA-damaged cells could block the depletion of NAD^+, thereby preserving ATP pools and facilitating ATP-dependent synthetic processes. To evaluate this possibility, we measured poly(ADP-ribose) levels in MNNG-treated cells to confirm that nicotinamide and benzamide could actually inhibit polymer synthesis at the cellular level and then measured the effect of these compounds on NAD^+ and ATP pools in the DNA-damaged cells.

Effect of Nicotinamide and Benzamide on Intracellular Poly(ADP-ribose) Levels. Figure 1 shows the levels of poly(ADP-ribose) measured in L1210 cells before and at specific time points after the cells were treated with 136 μM MNNG. Polymer levels are expressed in units of εRAdo, which is the fluorescent monomer subunit derived from poly(ADP-ribose), and measured by the HPLC and fluorescent detection technique outlined under Methods. Untreated cells contained 1.5

Table III: Effect of Nicotinamide on MNNG-Induced NAD^+ and ATP Depletion and DNA Repair Synthesis[a]

MNNG (μM)	nicotinamide	pmol of $NAD^+/10^6$ cells				pmol of ATP/10^6 cells				DNA synthesis (dpm [^{3}H]dTMP/10^6 cells in 6 h)
		0.5 h	1.0 h	3.0 h	6.0 h	0.5 h	1.0 h	3.0 h	6.0 h	
0	–	449	432	416	312	2900	3100	2500	2500	62 000
	+	559	551	638	524	3100	3200	3100	2000	64 000
13.6	–	286	203	234	162	3200	3100	3000	1900	64 000
	+	512	530	557	492	3100	3300	3000	2300	70 000
34	–	82	59	68	57	3000	1900	1800	1200	70 000
	+	441	313	353	335	3000	2900	2500	2000	70 000
68	–	53	26	9	6.5	2000	1600	451	134	40 000
	+	274	111	132	134	2800	2900	2500	1600	104 000
136	–	25	0.04	0.04	0.04	1500	920	180	70	31 000
	+	94	62	110	118	3300	2500	2000	1100	91 000

[a] Cultures were pretreated for 15 min with 10 mM hydroxyurea. At time zero, the indicated concentrations of MNNG were added and where indicated (+), the cultures were adjusted to contain 2 mM nicotinamide, and then [^{3}H]dThd was added to label DNA. At the indicated times, duplicate samples were removed, and NAD^+ and ATP were extracted and measured in duplicate as described under Methods. After 6 h in hydroxyurea-containing medium, the incorporation of [^{3}H]dTMP was measured as described under Methods.

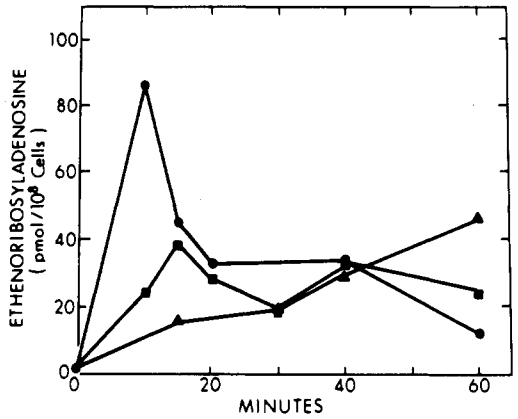

FIGURE 1: Effect of poly(ADP-ribose) polymerase inhibitors on intracellular poly(ADP-ribose) levels in MNNG-treated L1210 cells. All cultures were treated with 136 μM MNNG (●), and portions were adjusted to contain 2 mM nicotinamide (■) or 2 mM benzamide (▲). At the indicated times, between 5×10^7 and 2×10^8 cells were removed, and poly(ADP-ribose) was extracted and quantified as described under Methods.

pmol of ϵRAdo/10^8 cells. At 10 min after the cells were treated with 136 μM MNNG, polymer levels rose to 86 pmol of ϵRAdo/10^8 cells. By 60 min, polymer levels decreased to less than 13 pmol of ϵRAdo/10^8 cells. This rapid increase and decrease indicates that poly(ADP-ribose) is rapidly turned over in DNA-damaged cells. The addition of 2 mM nicotinamide to the culture immediately after MNNG treatment retarded the rapid increase in polymer levels. At 10 min, there was significantly less polymer in MNNG-treated cells incubated with nicotinamide than in cells treated with MNNG alone. While nicotinamide-treated cells were able to synthesize poly(ADP-ribose), they never achieved the levels seen at 10 min in cells treated with MNNG alone. The addition of 2 mM benzamide to MNNG-treated cells caused an even greater inhibition of poly(ADP-ribose) synthesis. At early times there was very little poly(ADP-ribose) in benzamide-treated cells; however, a steady increase in polymer levels occurred during the 60-min incubation. Similar to the observation in nicotinamide-treated cells, cells treated with benzamide never reached the levels found at 10 min in cells treated with MNNG alone. Determinations performed at 3 and 6 h after MNNG treatment showed that poly(ADP-ribose) levels had returned to base line and there was essentially no difference between the cells incubated in the presence or absence of the poly(ADP-ribose) polymerase inhibitors. Thus, the addition of 136 μM MNNG to L1210 cells caused transient increases in poly(ADP-ribose) levels of up to 57-fold greater than in controls, and the presence of either nicotinamide or benzamide greatly retarded this increase.

Nicotinamide Causes Altered NAD^+ and ATP Lowering in MNNG-Treated Cells. When NAD^+ levels were measured in cells treated with 136 μM MNNG (Table III), we found that rapid NAD^+ lowering occurred as expected from the increase in poly(ADP-ribose) levels and the rapid turnover of this polymer. When cells were incubated with 2 mM nicotinamide, NAD^+ lowering was much less rapid as anticipated from the ability of this agent to retard the increase in poly(ADP-ribose) levels. Thus the stimulation of [^{3}H]dTMP incorporation by nicotinamide was associated with retardation of poly(ADP-ribose) synthesis and partial preservation of the NAD^+ pool.

We subsequently examined the effect of nicotinamide on the NAD^+ and ATP pools over a wide range of MNNG concentrations. Table III shows that L1210 cells had initial levels of 449 pmol of NAD^+ and 2900 pmol of ATP per 10^6 cells. In control, untreated cells reasonably stable pool sizes were maintained during the 6 h of these experiments. The addition of 2 mM nicotinamide to the medium of undamaged cells resulted in some expansion of the NAD^+ pool but did not significantly alter the ATP pool. Furthermore, nicotinamide did not affect [^{3}H]dTMP incorporation in these undamaged cells. When the L1210 cells were treated with low doses of MNNG (13.6 and 34 μM), a dose-dependent NAD^+ lowering occurred, and modest reductions in ATP pools were also observed. For example, when cells were treated with 34 μM MNNG, NAD^+ levels fell to 18% of the initial control level (82 compared to 449 pmol/10^6 cells) within 30 min and were still 13% of controls by 6 h. The ATP levels remained normal for the first 30 min and only fell to 41% of the initial control level (1200 compared to 2900 pmol/10^6 cells) by 6 h. Under these conditions, the presence of nicotinamide had a sparing effect on the NAD^+ and ATP levels; however, even in the absence of nicotinamide, the NAD^+ and ATP pools were not as drastically depleted as they were when the cells were treated with high doses of MNNG.

When cells were treated with high doses of MNNG (68 or 136 μM), there was rapid NAD^+ lowering followed by drastic reductions in the ATP pool. For example, when cells were treated with 136 μM MNNG, NAD^+ levels fell to 6% of controls in 30 min and to less than 0.01% of controls by 6 h. ATP levels fell to 52% of controls in 30 min and to 2% of controls by 6 h. The presence of nicotinamide partially prevented these severe nucleotide depletions. For example, when nicotinamide was present in the medium of cells treated with 136 μM MNNG, NAD^+ levels fell to 21% of controls in 30

min and were maintained at 26% of controls after 6 h. In the presence of nicotinamide, ATP levels remained normal for 1 h and were still at 38% of controls by 6 h. Nicotinamide stimulated [^{3}H]dTMP incorporation 2.6-fold in cells treated with 68 μM MNNG and 2.9-fold in cells treated with 136 μM MNNG. Thus in the absence of DNA damage or at low doses of MNNG, ATP levels were not drastically depleted, and cells showed increasing rates of [^{3}H]dTMP incorporation in response to DNA damage. Under these conditions, the presence of 2 mM nicotinamide in the medium did not affect [^{3}H]-dTMP incorporation. Nicotinamide only stimulated [^{3}H]-dTMP incorporation when the level of MNNG-induced damage was sufficiently high to drastically deplete cellular NAD^+ and ATP pools.

These data clearly show that high doses of MNNG cause large increases in poly(ADP-ribose) synthesis that results in the rapid depletion of intracellular NAD^+ and ATP. The addition of poly(ADP-ribose) polymerase inhibitors results in decreased rates of poly(ADP-ribose) synthesis and decreased rates of NAD^+ lowering such that there are adequate NAD^+ levels to support ATP synthesis, which is required for DNA, RNA, and protein synthesis (Klein & Bonhoeffer, 1972; Chamberlin, 1974; Schimmel & Soll, 1979; Kurland, 1977). When the MNNG-induced increase in poly(ADP-ribose) synthesis was not sufficient to deplete NAD^+ and ATP levels, then addition of poly(ADP-ribose) polymerase inhibitors had no effect on the level of DNA synthesis.

It is interesting to note in Table III that increasing concentrations of MNNG up to 34 μM resulted in increasing levels of DNA repair synthesis. However, when MNNG concentrations were increased to 68 and 136 μM, DNA synthesis decreased to 40 000 and 31 000 dpm/10^6 cells in 6 h, respectively, compared to 70 000 dpm/10^6 cells at lower doses of MNNG. When cells were treated with high doses of MNNG and incubated with nicotinamide, the levels of DNA repair synthesis were elevated above those induced in cells treated with 13.6 or 34 μM MNNG. These results suggest that increasing concentrations of DNA-damaging agents cause increasing levels of DNA repair synthesis until the damage is sufficiently great to cause ATP depletion, which then limits the DNA repair process. Addition of nicotinamide to these culture systems preserved ATP levels and allowed DNA repair synthesis to increase to a level more appropriate to the degree of DNA damage.

Effect of Delaying Addition of Nicotinamdie to MNNG-Treated Cells. If the nicotinamide-stimulated DNA synthesis is mediated by preservation of the ATP pool sizes, then we would predict that as cells become progressively depleted of NAD^+ and ATP, nicotinamide should lose its ability to stimulate [^{3}H]dTMP incorporation. Table IV shows the incorporation of [^{3}H]dTMP observed in cell cultures to which nicotinamide was added at various times after the addition of 136 μM MNNG. When nicotinamide was added immediately following the MNNG, there was a 3.4–3.7-fold stimulation of [^{3}H]dTMP incorporation. When nicotinamide was added 2 h after MNNG, there was only a 1.9–2.1-fold stimulation of [^{3}H]dTMP incorporation. When nicotinamide was added 4 h after MNNG treatment, there was only a 1.1–1.3-fold stimulation of [^{3}H]dTMP incorporation. Thus, as the cells became progressively depleted of NAD^+ and ATP, the addition of nicotinamide became progressively less effective at stimulating [^{3}H]dTMP incorporation.

Table IV: Effect of Nicotinamide on DNA Synthesis following MNNG-Induced NAD^+ and ATP Depletion[a]

damage	addition	DNA synthesis (dpm [^{3}H]dTMP/10^6 cells in 6 h) for time of addition (h) 0	2	4
	Experiment 1			
136 μM MNNG	saline	35 000	36 000	36 000
136 μM MNNG	2 mM nicotinamide	120 000	70 000	46 000
	Experiment 2			
136 μM MNNG	saline	58 000	61 000	61 000
136 μM MNNG	2 mM nicotinamide	212 000	128 000	65 000

[a] Cultures were pretreated for 15 min with 10 mM hydroxyurea. At time zero, MNNG and [^{3}H]dThd were added to all cultures. At the indicated times, some cultures were adjusted to contain 2 mM nicotinamide. The incorporation of [^{3}H]dTMP was measured 6 h after addition of [^{3}H]dThd as described under Methods. Hydroxyurea was present during the entire labeling period.

Discussion

Previous studies from our laboratory have shown that poly(ADP-ribose) polymerase inhibitors stimulate [^{3}H]dTMP incorporation in DNA-damaged resting human lymphocytes and that this stimulation of [^{3}H]dTMP incorporation is proportional to the potency of the compounds as inhibitors of poly(ADP-ribose) polymerase (Sims et al., 1982; Jacobson, E. L., et al., 1980). Similar results have now been reported in resting human lymphocytes (Miwa et al., 1981; Bohr & Klenow, 1981) and in nonreplicating hepatocytes (Althaus et al., 1980). Our present studies show that nicotinamide and benzamide stimulate the level of DNA synthesis in rapidly growing L1210 cells that have been treated with DNA-damaging agents. Similar observations have also been recently reported for L1210 cells treated with Me_2SO_4 (Durkacz et al., 1981). Thus the stimulation of DNA synthesis by nicotinamide analogues only occurs in DNA-damaged cells and is independent of their replicative status.

Treatment of eukaryotic cells with DNA-damaging agents activates poly(ADP-ribose) polymerase (Berger et al., 1979, 1980; Jacobson, M. K., et al., 1980; Durkacz et al., 1980; Sudhakar et al., 1979; Skidmore et al., 1979; Benjamin & Gill, 1980), and we found that when L1210 cells were treated with 136 μM MNNG, there was a 57-fold increase in poly(ADP-ribose) levels. Maximal polymer levels occurred 10–15 min following MNNG treatment and then they rapidly declined, indicating rapid turnover in vivo. This rapid synthesis and turnover of poly(ADP-ribose) in DNA-damaged cells is similar to the processes recently described in 3T3 cells (Jaurez-Salinas et al., 1979) and normal human lymphocytes (Sims et al., 1982) and accounts for the rapid depletion of NAD^+ levels in DNA-damaged cells. When 2 mM nicotinamide or benzamide was present in the culture medium, polymer synthesis was retarded but not blocked entirely.

Our measurements of polymer levels reflect the accumulation of polymer at specific times, which is the balance between synthesis and degradation. The altered kinetics of polymer accumulation in nicotinamide- and benzamide-treated cells may reflect an effect on both synthesis and degradation. Our data show that although polymer levels peak at later times in cells treated with inhibitors, by 3 h the steady-state levels have returned to basal levels in 136 μM MNNG treated cells. This is also similar to our observations in intact lymphocytes (Sims et al., 1982) that poly(ADP-ribose) polymerase inhibitors retard the rate of poly(ADP-ribose) formation but do not completely prevent polymer synthesis.

Activation of poly(ADP-ribose) polymerase by DNA damage can cause the abrupt lowering of cellular NAD^+ pools (Jacobson, M. K., et al., 1980; Durkacz et al., 1980; Skidmore

et al., 1979; Goodwin et al., 1978), and polymerase inhibitors that retard the rate of polymer synthesis also slow down the rate of NAD^+ depletion according to their ability to inhibit the polymerase (Sims et al., 1982). When NAD^+ pools were severely depleted in L1210 cells, there was a subsequent drastic fall in their ATP pools. Thus the ATP pool fell to less than 3% of controls within 6 h of treatment with 136 μM MNNG. The presence of nicotinamide allowed the MNNG-treated cells to partially preserve their ATP pools such that by 6 h after treatment these cells still maintained 38% of their initial ATP levels.

In the present studies, we observed that poly(ADP-ribose) polymerase inhibitors stimulated DNA synthesis in DNA-damaged cells after high doses of MNNG but not after low doses. A similar result was recently reported by Durkacz et al. (1981), who showed that 3-aminobenzamide stimulated DNA repair synthesis in L1210 cells after high doses of Me_2SO_4 but not after low doses. These findings are consistent with our notion that the ability of the poly(ADP-ribose) polymerase inhibitors to stimulate DNA synthesis, after high doses of DNA-damaging agents, is due to their ability to retard the depletion of the NAD^+ pool, which has the effect of preserving the ATP pool and ATP-dependent functions.

There are several mechanisms by which the preservation of the ATP pool in DNA-damaged cells could stimulate [^{3}H]dTMP incorporation. One possibility is that higher ATP levels allow more [^{3}H]dThd to be phosphorylated, which increases the amount of dTTP available for DNA synthesis. However, this is clearly not the only mechanism since nucleotide-permeable lymphocytes to which nucleoside triphosphates are exogenously supplied also show stimulation of [^{3}H]dTMP incorporation in the presence of poly(ADP-ribose) polymerase inhibitors (Berger & Sikorski, 1980). This observation has also recently been confirmed in permeabilized hepatocytes (Althaus et al., 1982). Furthermore, other energy-requiring processes such as RNA and protein synthesis were also stimulated by treatment of DNA-damaged cells with poly(ADP-ribose) polymerase inhibitors. The ability of poly(ADP-ribose) polymerase inhibitors to stimulate RNA synthesis in DNA-damaged cells following MNU treatment was also recently shown by Taniguchi et al. (1982). We chose a 6-h labeling period to accentuate the differences in DNA and RNA synthesis in cells treated with MNNG in the presence and absence of nicotinamide and benzamide. In preliminary experiments where the pulse-labeling period was varied from 1 to 6 h, differences in the rate of dTMP and UMP incorporation were apparent after 1 h, and the differences became progressively greater with increased pulse length. Although we cannot rule out changes in specific activity of the precursor pools as contributing to the differences in incorporation, the fact that we see differences at 1 h when we would expect precursor pools to be least affected suggests that specific activity changes in the precursor pools cannot totally account for the differences we see in inhibitor-treated cells. In the case where protein synthesis was measured as incorporation of [^{3}H]Leu, it is clear that ATP was not only required for the synthesis of a phosphorylated precursor as in the conversion of dThd to dTTP. Thus the higher levels of ATP in the cells treated with poly(ADP-ribose) polymerase inhibitors apparently lead to higher levels of net DNA, RNA, and protein synthesis. This is in agreement with the demonstration that all three of these processes are ATP dependent (Klein & Bonhoeffer, 1972; Chamberlin, 1974; Schimmel & Soll, 1979; Kurland, 1977).

Studies on the function of poly(ADP-ribose) polymerase in the DNA repair process suggest that the enzyme may perform two different functions. As noted in the introduction, under conditions where cells are treated with relatively low doses of DNA-damaging agents, poly(ADP-ribose) synthesis appears to facilitate the DNA repair process since inhibition of poly(ADP-ribose) polymerase prevents cells from rejoining DNA strand breaks and resuming replicative DNA synthesis (Durkacz et al., 1980; Jacobson, E. L., et al., 1980; Berger & Sikorski, 1981). These inhibiors of poly(ADP-ribose) polymerase also potentiate the cytotoxic effects of DNA-damaging agents (Durkacz et al., 1980; Durrant & Boyle, 1982). In contrast, the present study suggests that at very high levels of DNA damage, poly(ADP-ribose) polymerase acts as a suicide enzyme to kill the cells before they have a chance to repair DNA damage. Thus in eukaryotes, high levels of DNA damage activate poly(ADP-ribose) polymerase to a degree that depletes cellular NAD^+ levels and subsequently depletes ATP levels, causing a decrease in energy-dependent functions and consequently leading to cell death. Since prokaryotes do not contain poly(ADP-ribose) polymerase (Hayaishi & Ueda, 1977), high levels of DNA damage cannot activate this suicide pathway to deplete cellular NAD^+ and ATP levels. Thus at comparably high levels of DNA damage, eukaryotic cells might die due to depletion of NAD^+ and ATP whereas prokaryotes might survive, although their high level of damage might lead to a high mutation frequency. Such a suicide mechanism would probably be of benefit to a multicellular organism where it would be preferable for cells with severely damaged DNA to die rather than risk repair with a high level of infidelity. In contrast, in prokaryotes, where a high mutation frequency could contribute to survival, such a suicide mechanism would not be desirable. This concept is further supported by previous studies on the use of poly(ADP-ribose) polymerase inhibitors in combination with high levels of DNA-damaging agents. For example, when rats were treated with streptozotocin, there was loss of pancreatic islet cell function, and at higher doses of streptozotocin, there was an increase in drug-related deaths (Rakieten et al., 1976; Schein et al., 1967). It was clearly shown that treatment with high doses of streptozotocin caused severe NAD^+ depletion and this depletion could be blocked by simultaneous administration of nicotinamide (Kazumi et al., 1978). Furthermore, when nicotinamide was administered along with high doses of streptozotocin, there was an increase in survival; however, a high percentage of the survivors had pancreatic islet cell tumors (Kazumi et al., 1978). Thus, a suicide function of poly(ADP-ribose) polymerase may serve as a protective mechanism against the accumulation of mutated cells after heavy mutagenic exposure.

Registry No. MNNG, 70-25-7; NAD, 53-84-9; ATP, 56-65-5; poly(ADP-ribose) polymerase, 9055-67-8; nicotinamide, 98-92-0; benzamide, 55-21-0.

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Nuclear Overhauser Effect as a Tool for the Complete Assignment of Nonexchangeable Proton Resonances in Short Deoxyribonucleic Acid Helices†

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ABSTRACT: A new strategy for the assignment of nonexchangeable proton resonances in oligonucleotide duplexes is presented and used to interpret the spectra of the oligonucleotide helix d(CpGpCpGpCpG)·d(CpGpCpGpCpG) in low salt (B form). This procedure is based on the use of sequential homodecoupling (1D) or COSY (2D) for the interconnection of the sugar resonances pertaining to the same residue and on the measurement of nuclear Overhauser effect (NOE) (1D or 2D) between critically located protons in order to establish the connectivity between the base protons and the sugar protons, as well as between consecutive base–sugar residues. The assignment performed by this method was found in perfect agreement with the one made previously by the incremental procedure [Cheng, D. M., Kan, L.-S., Frechet, D., Ts'o, P. O. P., Uesugi, S., Shida, T., & Ikehara, M. (1983) *Biopolymers* (in press)]. Also, this method is demonstrated to be applicable to DNA short helices containing A·T base pairs.

Nuclear magnetic resonance (NMR) is one of the most powerful methods currently available for studying the conformation of nucleic acids in solution. Short oligonucleotide duplexes have been extensively used as models in such studies [Cheng et al., 1983; Patel et al., 1982a,b; Kan et al., 1982; Kearns et al., 1981; Tran-Dinh et al., 1982a,b; Cross & Crothers, 1971; Pardi et al., 1981; Olsthoorn et al. (1980) and references cited therein]. The conformation of these short helices in solution is sufficiently close to that of native DNA to allow their recognition by enzymes (Miller et al., 1982), yet their small size makes them suitable for obtaining high-resolution NMR spectra.

In early studies, base protons, imino protons, and sugar protons were analyzed on a group basis (Cross & Crothers,

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