Poly(adenosinediphosphoribose) Polymerase Inhibitors Stimulate Unscheduled Deoxyribonucleic Acid Synthesis in Normal Human Lymphocytes†

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ABSTRACT: Thirty-three analogues of the nicotinamide and adenine portions of NAD^+ were evaluated for (1) their effectiveness as inhibitors of poly(adenosinediphosphoribose) polymerase in nucleotide permeable lymphocytes, (2) their ability to interfere with NAD^+ lowering in DNA-damaged cells, (3) their ability to inhibit accumulation of poly(adenosinediphosphoribose) in DNA-damaged cells, and (4) their ability to stimulate unscheduled DNA synthesis in DNA-damaged cells. Poly(adenosinediphosphoribose) polymerase was effectively inhibited by six-membered aromatic ring compounds containing a carboxamide group such as nicotinamide, picolinamide, 5-methylnicotinamide, benzamide, 3-aminobenzamide, and 5-methoxybenzamide. Compounds containing a carboxylic acid group rather than a carboxamide group did not inhibit poly(adenosinediphosphoribose) polymerase. Purine analogues such as theobromine and theophylline were also effective inhibitors of poly(adenosinediphosphoribose) polymerase. The ability of these compounds to inhibit poly(adenosinediphosphoribose) polymerase in nucleotide permeable cells correlated with their ability to retard NAD^+ lowering in vivo in cells treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. None of the polymerase inhibitors examined were able to completely prevent the fall of NAD^+ levels induced by treating cells with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. However, the most effective polymerase inhibitors caused the greatest retardation in the rate of NAD^+ lowering. Within 10 min of treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, cells showed a 30–40-fold increase in poly(adenosinediphosphoribose) levels. Inhibitors of poly(adenosinediphosphoribose) polymerase delayed the time at which maximal polymer levels were reached; however, they did not completely block its synthesis. Thus, the compounds that were the most effective polymerase inhibitors in vitro were also the most potent in retarding NAD^+ lowering in vivo and the most potent in delaying poly(adenosinediphosphoribose) accumulation in vivo. These compounds were also found to be the most effective stimulators of unscheduled DNA synthesis. For example, unscheduled DNA synthesis in UV-irradiated cells was stimulated 2-fold by treatment with 2 mM benzamide, nicotinamide, or 3-aminobenzamide.

The synthesis of poly(ADP-ribose)[1] by the chromatin-bound enzyme poly(ADP-ribose) polymerase (Hilz & Stone, 1976; Hayaishi & Ueda, 1977) is stimulated by exposure of cells to DNA-damaging agents such as X irradiation, γ irradiation, bleomycin, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), and UV irradiation (Benjamin & Gill, 1980; Goodwin et al., 1978; Miller, 1977; Jaurez-Salinas et al., 1979; Berger et al., 1979). The substrate for poly(ADP-ribose) polymerase is NAD^+ (Hayaishi & Ueda, 1977), and the intracellular levels of NAD^+ are acutely lowered by some treatments that cause DNA damage (Jacobson, M. K., et al., 1980; Rankin et al., 1980). M. K. Jacobson et al. (1980) have recently shown that the lowering of the NAD^+ pool that occurs when 3T3 cells are treated with MNNG is due to its increased consumption and not due to any decrease in NAD^+ synthesis. It has also been shown that poly(ADP-ribose) is rapidly synthesized and degraded during this period of rapid NAD^+ lowering (Jaurez-Salinas et al., 1979). A requirement for poly(ADP-ribose) synthesis in the DNA repair process has recently been suggested by the demonstration that inhibitors of poly(ADP-ribose) polymerase prevent the rejoining of DNA strand breaks occurring in L1210 cells after treatment with dimethyl sulfate (Durkacz et al., 1980) and by the finding that 3T3 cells made NAD^+ deficient by nicotinamide starvation lose the capacity to repair MNNG-induced DNA damage (Jacobson, E. L., et al., 1980). In the latter case, addition of nicotinamide to the culture medium resulted in restoration of normal NAD levels as well as the capacity to perform DNA repair.

Normal human lymphocytes are similar to the cell types noted above in that poly(ADP-ribose) polymerase activity and DNA repair synthesis are stimulated by treatment of the cells with UV irradiation, MNNG, 2-(*N*-acetoxyacetylamino)-fluorene (AAAF), and bleomycin (Berger et al., 1979). In addition, acute lowering of lymphocyte NAD^+ levels has been shown to occur following treatment with MNNG, AAAF, methyl methanesulfonate, and benzo[*a*]pyrene (Rankin et al., 1980). Recently, we showed that treatment of lymphocytes with DNA-damaging agents followed by incubation in medium containing 2 mM nicotinamide resulted in a 2-fold stimulation in the level of unscheduled DNA synthesis (Berger & Sikorski, 1980). Since nicotinamide is a precursor for the synthesis of NAD^+ as well as an inhibitor of poly(ADP-ribose) polymerase, it is possible that either or both of these properties may be responsible for the ability of nicotinamide to stimulate DNA repair in DNA-damaged lymphocytes. To determine whether the stimulation of unscheduled DNA synthesis could be associated with inhibition of poly(ADP-ribose) polymerase, we screened a series of 33 compounds for their ability to inhibit

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[1] Abbreviations: poly(ADP-ribose), poly(adenosinediphosphoribose); MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; UV, ultraviolet; NAD^+, nicotinamide adenine dinucleotide, oxidized form; AAAF, 2-(*N*-acetoxyacetylamino)fluorene; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; Me_2SO, dimethyl sulfoxide; dThd, thymidine; Tris, tris(hydroxymethyl)aminomethane; Mops, 3-(*N*-morpholino)propanesulfonic acid; HPLC, high-performance liquid chromatography; dTMP, thymidine 5'-monophosphate; L-PHA, L-phytohemagglutinin.

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poly(ADP-ribose) polymerase in nucleotide-permeable cells, then examined their ability to interfere with NAD^+ lowering and poly(ADP-ribose) synthesis in vivo, and then compared these results with their ability to stimulate unscheduled DNA synthesis in DNA-damaged cells.

Experimental Procedures

Materials

Nicotinamide, nicotinic acid, 6-aminonicotinamide, 6-aminonicotinic acid, *N*-methylnicotinamide, 1-methylnicotinamide chloride, theobromine, benzoic acid, caffeine, *N,N*-diethylnicotinamide, picolinic acid, isoniazid, pyrazinamide, hydroxyurea, and L-phytohemagglutinin were purchased from Sigma Chemical Co. (St. Louis, MO). 3-Aminomethylpyridine, *m*-aminobenzoic acid, *m*-methoxybenzamide, 2-pyrazinecarboxylic acid, 2,3-pyrazinedicarboxamide, pyrazine, nipecotamide, 2,3-pyrazinedicarboxylic acid, isonipecotamide, isonicotinic acid, and benzamide were purchased from Aldrich (Milwaukee, WI). Pyrazinamide, *o*-aminobenzamide, *p*-aminobenzamide, and *m*-aminobenzamide were purchased from Pfaltz and Bauer, Inc. (Stanford, CT). [*adenosine*-U-^{14}C]NAD^+, sp act. 534 mCi/mmol, [*methyl*-^{3}H]thymidine, sp act. 73.6 Ci/mmol, and [*adenine*-2,8-^{3}H]NAD^+, sp act. 3 Ci/mmol, were purchased from New England Nuclear (Boston, MA). Theophylline was purchased from Calbiochem-Behring Corp. (La Jolla, CA). Whatman GF/C filters and Me_2SO were purchased from Fisher (Fairlawn, NJ). 5-Methylnicotinamide was the generous gift of Dr. Kurt Gurzon of the Eli Lilly Research Laboratories (Indianapolis, IN).

Methods

Lymphocyte Preparation and Determination of DNA Repair Synthesis. Normal human lymphocytes were isolated from peripheral blood (Mendelsohn et al., 1971) and were suspended at 2×10^6 cells/mL in complete medium composed of α-modified Eagles 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. For UV irradiation, cells were collected by centrifugation at 800*g* for 5 min and resuspended in phosphate-buffered saline at 2×10^6/mL. Ten-milliliter aliquots of cell suspensions were spread in 150 mm diameter plastic Petri dishes and UV irradiated; cells were then collected by centrifugation as above and resuspended in complete medium at 1×10^6/mL as previously described (Berger et al., 1979). Control cells were subjected to the same procedure except that they were not irradiated. For treatment with MNNG, cells were suspended in complete medium at 1×10^6/mL and were made 136 μM in MNNG and 0.2% in dimethyl sulfoxide (Me_2SO) by the addition of MNNG which was dissolved in Me_2SO just prior to addition to cell cultures. Control cells were made 0.2% in Me_2SO. Two-milliliter samples of the UV-irradiated, MNNG, and control cell cultures were immediately distributed into sterile culture tubes and adjusted to 10 mM in hydroxyurea and to the indicated concentration of nicotinamide analogue or purine analogue. After 30 min at 37 °C, [^{3}H]dThd (sp act. 73.6 Ci/mmol) was added to a final concentration of 10 μCi/mL, and incubations were continued for 6 h at 37 °C. Thymidine incorporation was stopped by adding 10 mL of ice-cold 0.9% saline to the tubes, and cells were collected by centrifugation at 1210*g* for 7 min at 4 °C. The medium was aspirated, and 5 mL of ice-cold 5% trichloroacetic acid was added. Tubes were held in an ice water bath for 30 min. Precipitates were briefly sonicated and were then collected on GF/C glass fiber filters. Tubes were rinsed 4 times with 5 mL of ice-cold 5% trichloroacetic acid and once with 5 mL of ice-cold 95% ethanol. Filters were placed in scintillation vials and dried. Fifty microliters of water and 0.5 mL of Protosol were added. After an overnight solubilization in closed vials, 8 mL of scintillation fluid [4.2 g of 2,5-diphenyloxazole (PPO) and 0.525 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene (POPOP) per L of toluene] was added and radioactivity determined by scintillation counting.

Determination of NAD Lowering. At selected times after DNA-damaging treatments, cells were collected by centrifugation at 2250*g* for 3 min at 4 °C, the medium was aspirated, and the cell pellets were acidified with 200 μL of 0.35 N perchloric acid. After 15 min at 4 °C, acid extracts were neutralized with 25 μL of a solution containing 1 M Imid base, 2.1 M potassium hydroxide, and 0.5 M potassium chloride. NAD^+ was measured by enzymatic cycling techniques as previously described (Kato et al., 1973).

Poly(ADP-ribose) Polymerase Assays. The effect of nicotinamide analogues and purine analogues on poly(ADP-ribose) polymerase was measured in L-phytohemagglutinin (L-PHA) stimulated lymphocytes. Lymphocytes in complete medium were incubated with L-PHA at a final concentration of 2 μg/mL for 3 days. Stock solutions of nicotinamide analogues and purine analogues were dissolved in 10 mM Tris-HCl and then adjusted to a final pH of 7.8. 6-Mercaptopurine was dissolved in Me_2SO. Enzyme activity was measured in nucleotide-permeable cells by incorporation of the ADP-ribose portion of [^{3}H]NAD^+ into the acid-insoluble form as previously described (Berger et al., 1979).

Determination of Poly(ADP-ribose) Levels. After various incubation periods, samples of control and DNA-damaged cells were transferred to sterile 50-mL plastic centrifuge tubes and were collected by centrifugation at 1990*g* for 5 min at 4 °C. The medium was quickly aspirated, and the cells were precipitated by immediately adding 5 mL of ice-cold 20% trichloroacetic acid. After 30 min at 4 °C, precipitates were collected by centrifugation at 1990*g* for 5 min at 4 °C. Pellets were washed with an additional 5 mL of ice-cold 20% trichloroacetic acid and the precipitates collected as above. Poly(ADP-ribose) was extracted and quantified by a modification of the method of Sims et al. (1980). Trichloroacetic acid precipitates were dissolved in 6 M guanidine hydrochloride–0.1 M potassium phosphate buffer, pH 8.6, and poly(ADP-ribose) was selectively extracted by utilizing dihydroxyboryl-Sepharose affinity chromatography. The solubilized extracts of 2.5×10^7 to 1×10^8 cells were passed over 0.8-mL dihydroxyboryl-Sepharose columns, and guanidine buffer was removed as previously described (Sims et al., 1980). The unique compound ribosyladenosine, derived only from poly(ADP-ribose), was obtained by digesting the polymer, adsorbed to the dihydroxyboryl-Sepharose column, to nucleosides with snake venom phosphodiesterase and bacterial alkaline phosphatase.

Bacterial alkaline phosphatase (1000 units) was preincubated with 100 units of venom phosphodiesterase in 90 mM Mops buffer, pH 8.6, containing 20 mM $MgCl_2$ at 37 °C for 5 h to degrade contaminating nucleic acids. This digest was dialyzed 6 times against 100 volumes of the same buffer by using dialysis tubing with a 3500 molecular weight cutoff. Before being applied to the dihydroxyboryl-Sepharose column, additional venom phosphodiesterase was added so that the final concentrations were 24.6 units/mL bacterial alkaline phosphatase and 11.6 units/mL venom phosphodiesterase. Each 0.8-mL column was treated with 285 μL of this enzyme preparation and incubated at 37 °C for 3 h. Nucleosides were eluted with 1.6 mL of 10 mM sodium citrate buffer, pH 4.5.

After removal of the enzymes by precipitation with 20% trichloroacetic acid followed by extraction of the acid with diethyl ether, the unique fluorescent compound 1,N^6-ethenoribosyladenosine was formed by incubating nucleosides with 20 mM chloroacetaldehyde in 100 mM sodium citrate buffer, pH 4.5, for 8 h. Excess chloroacetaldehyde was extracted with diethyl ether, and ethenoribosyladenosine was separated from other fluorescent compounds by high-pressure liquid chromatography on a Varian 4 mm i.d. × 300 mm, MCH-10, reversed-phase column coupled to a 4 mm i.d. × 50 mm guard column packed with 40-μm Vydac reversed-phase packing material. Fluorescent derivatives were eluted isocratically with 7 mM ammonium formate (pH 5.8)–methanol, 88:12 (v/v), by utilizing a Varian Model 5000 HPLC equipped with a heating block maintained at 25 °C. Ethenoribosyladenosine was quantified by fluorescence detection by using a Varian Fluorichrom fluorometer equipped with a 220-nm interference filter for excitation and a Corning 3-75, 370-nm, cutoff filter and a Corning 5-58, 410-nm, band-pass filter for emission. The recovery of the polymer was routinely 70–80% as judged by the recovery of radioactively labeled nucleosides from cell extracts containing [^{14}C]poly(ADP-ribose). Radioactively labeled polymer used in the recovery controls was synthesized in nucleotide-permeable human lymphocytes and had an average chain length of 14 ADP-ribose units.

A standard curve for the detection of ethenoribosyladenosine was constructed by plotting fluorescence peak height vs. picomoles of authentic [^{14}C]ethenoribosyladenosine. For construction of the standard curve, authentic radiolabeled ribosyladenosine (sp act. 2 μCi/μmol) was prepared by incubation of partially purified sheep thymus poly(ADP-ribose) polymerase (Petzold et al., 1981) with 0.5 mM [*adenosine*-U-^{14}C]NAD$^+$ (sp act. 2 μCi/μmol) for 5 min at 37 °C in a 1-mL reaction containing 100 mM Tris-HCl, pH 8.0, 1 mM dithiothreitol, 4 mM $MgCl_2$, 100 μg of calf thymus DNA, and 100 μg of histone H1. The reaction was stopped by transferring the tube to an ice water bath and addition of ice-cold 100% trichloroacetic acid to a final concentration of 20%. Acid-precipitable material was collected by centrifugation at 12800*g* for 15 min. The pellet was washed twice with 1 mL of ice-cold 20% trichloroacetic acid and then dissolved in 6 M guanidine hydrochloride containing 0.1 M potassium phosphate buffer, pH 8.6. The polymer was adsorbed to dihydroxyboryl-Sepharose and digested to nucleosides as described above. Nucleoside mixtures were incubated with chloroacetaldehyde to form the etheno derivatives; ethenoribosyladenosine was obtained after separation of derivatives by reversed-phase chromatography as described above. This derivative was identical in chromatographic properties, UV spectrum, and fluorescence emission with the standard ethenoribosyladenosine used previously (Sims et al., 1980). The detection limit was 1 pmol of ethenoribosyladenosine.

Results

Effect of Nicotinamide on NAD Metabolism and DNA Repair Synthesis in Control and DNA-Damaged Resting Lymphocytes. Table I shows the changes that occur in DNA synthesis in resting or DNA-damaged lymphocytes when 2 mM nicotinamide is added to the culture medium. In the first column under donor 1, it can be seen that addition of 2 mM nicotinamide had very little effect on the rate of DNA synthesis in control, undamaged cells. Treatment of these cells with UV irradiation produced an increase in [^{3}H]dTMP incorporation from 40 900 to 56 200 dpm. When the UV-irradiated cells were incubated in the presence of 2 mM nicotinamide, there was a further increase in the level of DNA synthesis from 56 200 to 119 400 dpm. To further evaluate that portion of the DNA synthesis that was attributable to replicative and that due to repair synthesis, we incubated the cells in 10 mM hydroxyurea to suppress replicative synthesis. While several recent reports have indicated some suppressive effects of hydroxyurea (Collins et al., 1977; Cornelius, 1978; Francis et al., 1979), this high concentration was purposely selected to give maximal suppression of replicative DNA synthesis. As shown in the second column under donor 1, the presence of 10 mM hydroxyurea produced marked suppression of DNA synthesis in the control, undamaged cells. The data in the second column also show that in the control undamaged cells, the presence of nicotinamide did not interfere with the ability of hydroxyurea to suppress replicative DNA synthesis. When the cells of donor 1 were damaged by UV irradiation and incubated with 10 mM hydroxyurea, there was only a small suppression of DNA synthesis. More importantly, 10 mM hydroxyurea had essentially no effect on the ability of the nicotinamide to induce the 2-fold stimulation in the level of DNA synthesis that occurred subsequent to DNA damage. Thus, under the stringent conditions of 10 mM hydroxyurea, as well as in its absence, 2 mM nicotinamide caused a marked increase in the unscheduled DNA synthesis that occurred in response to DNA damage. Similar results were obtained with cells from donor 2 in which the DNA-damaging treatment was 136 μM MNNG. Again in the control, undamaged cells, the addition of 2 mM nicotinamide had no effect on the level of DNA synthesis. After the cells were treated with MNNG, the subsequent level of DNA synthesis was less than that which occurred in the undamaged cells. This reduced level of DNA synthesis reflects the suppression of replicative DNA synthesis brought about by the DNA damage and the associated stimulation of DNA repair synthesis. Incubation of the MNNG-treated cells in the presence of 2 mM nicotinamide resulted in a 2-fold increase in the level of DNA synthesis even in the absence of hydroxyurea. The second column under donor 2 shows the results when the same series of cells were examined in the presence of 10 mM hydroxyurea. The 10 mM hydroxyurea drastically suppressed the level of DNA synthesis in the undamaged cells, but it had negligible effects on the unscheduled DNA synthesis that occurred in the MNNG-

Table I: Effect of Nicotinamide on Unscheduled DNA Synthesis in Human Lymphocytes

	DNA synth [dpm of [^{3}H]dTMP (10^6 cells)$^{-1}$ (6 h)$^{-1}$]			
	donor 1 [b,d]		donor 2 [c,d]	
incubation conditions [a]	−HU	+HU	−HU	+HU
control (undamaged)	40 900	5 100	24 500	3 700
control + 2 mM nicotinamide	35 500	5 500	23 400	4 090
DNA damage	56 200	45 400	11 100	9 820
DNA damage + 2 mM nicotinamide	119 000	99 300	22 600	20 000

[a] Freshly prepared human lymphocytes were incubated in growth medium which contained 8 μM nicotinamide or was supplemented to contain 2 mM nicotinamide where indicated. Control cells received no further treatment. [b] DNA-damaged cells were subjected to 20 J/m^2 UV irradiation for the cells from donor 1. [c] DNA-damaged cells were subjected to 136 μM MNNG and 0.2% Me_2SO for the cells from donor 2. Control, undamaged cell cultures were also adjusted to contain 0.2% Me_2SO. [d] Hydroxyurea was added to the indicated cultures (+HU) at a final concentration of 10 mM. The reactions designated −HU had no hydroxyurea added to the medium. Radioactive thymidine incorporation into acid-precipitable material was measured during a 6-h incubation period as described under Methods.

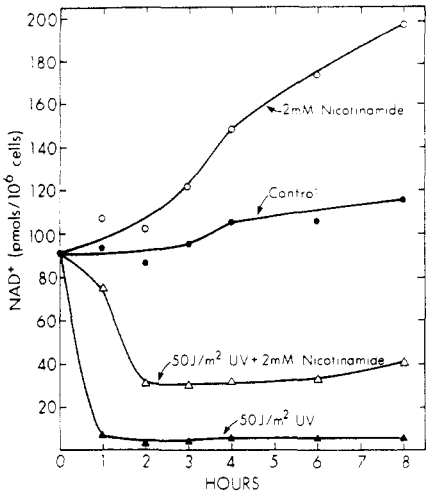

FIGURE 1: Effect of nicotinamide on NAD^+ pool size in normal human lymphocytes. At various times, duplicate samples of cell suspension were removed, and NAD^+ was extracted and assayed in duplicate as described under Methods. Results are presented as the means of assays performed in quadruplicate. Symbols for resting lymphocytes: no UV and no added nicotinamide (●); no UV plus 2 mM nicotinamide (○); 50 J/m^2 UV irradiation (▲); 50 J/m^2 UV irradiation plus 2 mM nicotinamide (△).

treated cells. More importantly, the presence of 10 mM hydroxyurea did not alter the ability of nicotinamide to stimulate DNA synthesis in DNA-damaged cells. In these experiments, 10 mM hydroxyurea was useful to suppress replicative DNA synthesis so that the component of DNA synthesis that occurred in response to DNA damage was more clearly discernible. Similar results were obtained in the presence and absence of hydroxyurea by using cells from six additional donors with nicotinamide and several other analogues. Further studies to evaluate the stimulation of DNA repair synthesis by nicotinamide analogues were therefore conducted in 10 mM hydroxyurea to eliminate the contribution of replicative synthesis. As indicated under Discussion, additional studies have now been published in several other cell systems to demonstrate that nicotinamide and its analogues stimulate the repair mode of DNA synthesis.

Figure 1 shows the effect of nicotinamide on the NAD^+ levels in normal and DNA-damaged cells in the presence and absence of 2 mM nicotinamide. When freshly isolated lymphocytes were incubated in complete medium, the cellular NAD^+ pool showed a slight increase over 8 h. When lymphocytes were incubated in medium containing 2 mM nicotinamide, there was a 2-fold increase in NAD^+ pools over 8 h. When lymphocytes were UV irradiated, there was a rapid lowering of NAD^+ to 5% of controls by 2 h. When UV-irradiated cells were incubated in the presence of 2 mM nicotinamide, a decrease occurred in the NAD^+ pool size but the rate of NAD^+ lowering and the extent of the lowering was less than when UV-irradiated cells were incubated in medium in the absence of added nicotinamide. This slower rate of NAD^+ lowering could be due to the ability of nicotinamide to stimulate NAD^+ synthesis or due to its ability to inhibit NAD^+-consuming enzymes such as poly(ADP-ribose) polymerase. To distinguish between these possible effects and to determine whether the stimulation of DNA repair is correlated with the ability to stimulate NAD^+ synthesis or to inhibit poly(ADP-ribose) synthesis, we surveyed a number of nicotinamide, benzamide, pyrazinamide, and purine analogues for their ability to inhibit poly(ADP-ribose) polymerase in nucleotide permeable cells. We then examined these compounds for their ability to inhibit poly(ADP-ribose) polymerase in vivo by measuring their effects on NAD levels and by using a sensitive chemical assay to measure their effects on polymer levels. The ability of these compounds to inhibit poly(ADP-ribose) polymerase was then compared with their ability to stimulate unscheduled DNA synthesis.

Poly(ADP-ribose) Polymerase Inhibition by NAD Analogues. (1) Inhibition by Nicotinamide Analogues. Figure 2 shows the structures of the compounds tested for their ability to inhibit poly(ADP-ribose) polymerase. These compounds are arranged from left to right and from top to bottom within each heading in order of decreasing ability to inhibit polymerase activity. Of the pyridine analogues tested, nicotinamide (compound **1**) and picolinamide (compound **2**) were the most effective polymerase inhibitors, causing 89 and 86% inhibition, respectively, as shown in Table II. Some structural alterations of these compounds decreased their potency as polymerase inhibitors while other modifications completely abolished their ability to inhibit polymerase activity. Movement of the carboxamide group to position 4 of the pyridine ring as in the case of isonicotinamide (compound **5**) decreased the capacity of the analogue to inhibit the polymerase to 69%. Modification at the carboxamide group of either nicotinamide or picolinamide had profound effects on the ability to inhibit the polymerase. A progressive decrease in capacity to inhibit the polymerase was observed when the carboxamide group of nicotinamide was altered as in 3-acetylpyridine (compound **7**) or blocked as in *N*-methylnicotinamide (compound **10**) and in *N*-diethylnicotinamide (compound **11**). Replacement of the carboxamide group with a carboxyl group as in nicotinic acid (compound **12**) resulted in complete loss of ability to inhibit the polymerase. Similar effects were seen with other pairs of compounds. 6-Aminonicotinamide (compound **4**) inhibited the polymerase 72% while 6-aminonicotinic acid (compound **15**) was unable to cause any inhibition. Similarly, isonicotinamide (compound **5**) inhibited the polymerase 69% while isonicotinic acid (compound **14**) did not cause any inhibition. These data demonstrate that an intact carboxamide group is required at positions 2, 3, or 4 of the pyridine ring for good polymerase inhibition.

Other modifications of the nicotinamide moiety of NAD were also examined for their abilities to inhibit poly(ADP-ribose) polymerase. Substitution at ring positions 5 and 6 had relatively little effect on inhibitory potency; for example, 5-methylnicotinamide (compound **3**) and 6-aminonicotinamide (compound **4**) gave 83 and 72% inhibition, respectively. Substitution of a methyl group on the ring nitrogen as in 1-methylnicotinamide (compound **6**) reduced inhibition of the polymerase to 29%. It was striking that polymerase inhibition was abolished when the ring system was completely saturated. For example, nicotinamide (compound **1**) showed 89% inhibition while nipecotamide (compound **16**) showed 0% inhibition; isonicotinamide (compound **5**) showed 69% inhibition while isonipecotamide (compound **17**) showed 0% inhibition. Thus, an aromatic ring system appears to be required for effective polymerase inhibition.

(2) Inhibition by Benzamide Analogues. Benzamide (compound **18**), 3-aminobenzamide (compound **19**), and 3-methoxybenzamide (compound **20**) caused 96% inhibition of enzyme activity and were the most potent inhibitors of poly(ADP-ribose) polymerase. The results with the benzamide analogues show that a ring nitrogen is not necessary for good polymerase inhibition. This series of compounds also showed similar trends of inhibition to those seen in the pyridine series in that a carboxamide group is required for potent inhibition. For example, benzamide (compound **18**) showed 96% inhib-

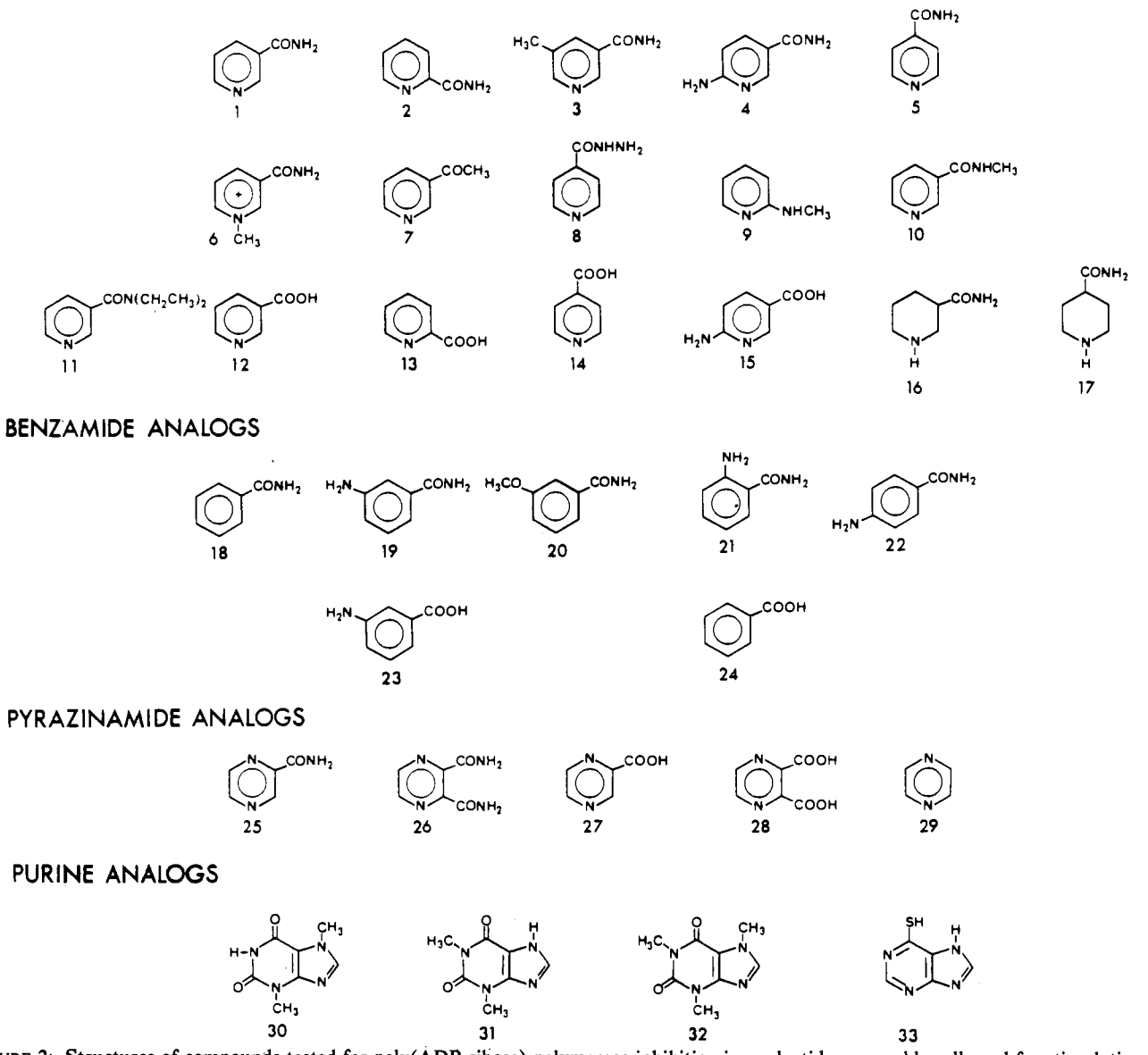

FIGURE 2: Structures of compounds tested for poly(ADP-ribose) polymerase inhibition in nucleotide-permeable cells and for stimulation of unscheduled DNA synthesis in vivo. Compounds are identified by referring to the numbers in the first column in Table II.

ition while benzoic acid (compound **24**) showed 0% inhibition and 3-aminobenzamide (compound **19**) showed 96% inhibition while 3-aminobenzoic acid (compound **23**) showed 0% inhibition. Substitution of the benzene ring with amino groups at position 2 or 4 caused a slight decrease of inhibitory potency. For example, benzamide (compound **18**) and 3-aminobenzamide (compound **19**) showed 96% inhibition while 2-aminobenzamide (compound **21**) and 4-aminobenzamide (compound **22**) showed 75 and 71% inhibition, respectively.

(*3*) *Inhibition by Pyrazinamide Analogues.* Studies with the pyrazinamide series of analogues showed that the presence of a pair of ring nitrogens, para to each other, did not interfere with the polymerase inhibition. Studies with these analogues showed again that the carboxamide group is essential for polymerase inhibition. For example, pyrazinamide (compound **25**) showed 78% inhibition of polymerase activity while pyrazinecarboxylic acid (compound **27**) showed 0% inhibition; pyrazine-1,2-dicarboxamide (compound **26**) showed 15% inhibition while pyrazine-1,2-dicarboxylic acid (compound **28**) showed no polymerase inhibition.

It can also be seen that a bulky substituent ortho to the carboxamide group decreases inhibitory potency since pyrazinamide (compound **25**) showed 78% inhibition of polymerase activity while pyrazine-1,2-dicarboxamide (compound **26**) was not very effective as a polymerase inhibitor.

(*4*) *Inhibition by Purine Analogues.* Purine analogues were also tested for polymerase inhibition. Theophylline (compound **31**) and theobromine (compound **30**) were good polymerase inhibitors while caffeine (compound **32**) and 6-mercaptopurine (compound **33**) were much less effective. These compounds presumably inhibit poly(ADP-ribose) polymerase activity by competing for a different portion of the NAD binding sight on the enzyme than do the nicotinamide analogues.

Poly(ADP-ribose) Synthesis in Resting Lymphocytes. If analogues which inhibit the polymerase in vitro are capable of inhibiting the polymerase in vivo, then it should be possible to show that these analogues interfere with the lowering of intracellular NAD^+ levels and the increase in poly(ADP-ribose) levels that occur when cells are treated with DNA-damaging agents. Figure 3 shows that NAD^+ levels were rapidly lowered by MNNG treatment. This lowering was not affected by nicotinic acid, benzoic acid, or pyrazinecarboxylic acid. These compounds did not inhibit poly(ADP-ribose) polymerase. The rate of NAD^+ lowering was slower in

Table II: Inhibition of Poly(ADP-ribose) Polymerase and Stimulation of Unscheduled DNA Synthesis by Analogues of Various Parts of the NAD^+ Molecule

no.[a]	compd[b]	polymerase act.[c] (% inhibn)	unscheduled synth[d] (% stim)
	control	0	100
	pyridine analogues		
1	nicotinamide	89	216
2	picolinamide	86	160
3	5-methylnicotinamide	83	175
4	6-aminonicotinamide	72	126
5	isonicotinamide	69	142
6	1-methylnicotinamide	29	98
7	3-acetylpyridine	27	125
8	isoniazid	14	99
9	2-(methylamino)pyridine	6	110
10	*N*-methylnicotinamide	2	113
11	*N*-diethylnicotinamide	1	93
12	nicotinic acid	0	100
13	picolinic acid	0	100
14	isonicotinic acid	0	99
15	6-aminonicotinic acid	0	100
16	nipecotamide	0	102
17	isonipecotamide	0	99
	benzamide analogues		
18	benzamide	96	186
19	3-aminobenzamide	96	215
20	3-methoxybenzamide	96	206
21	2-aminobenzamide	75	116
22	4-aminobenzamide	71	124
23	3-aminobenzoic acid	0	85
24	benzoic acid	0	93
	pyrazinamide analogues		
25	pyrazinamide	78	141
26	pyrazinedicarboxamide	15	106
27	pyrazinecarboxylic acid	0	96
28	pyrazinedicarboxylic acid	0	95
29	pyrazine	0	94
	purine analogues		
30	theobromine	81	159
31	theophylline	89	121
32	caffeine	35	97
33	6-mercaptopurine	16	122

[a] Compound numbers refer to structures of compounds shown in Figure 2. [b] Compounds were present in the assay system or culture medium at a concentration of 2 mM except theobromine which was 1 mM. [c] Polymerase activity was measured in nucleotide-permeable cells following 3 days of stimulation by 2 μg/mL L-phytohemagglutinin. Results are presented as the means of assays performed in duplicate or triplicate. Poly(ADP-ribose) polymerase activity in control cells, measured as incorporation of [^{3}H]ADPR, was 5100 dpm $(10^6$ cells$)^{-1}$ 30 min^{-1}. [d] Unscheduled DNA synthesis was measured in lymphocytes exposed to 20 J/m^2 UV irradiation. Results are presented as the means of assays performed in triplicate, and the relative stimulation is characteristic of experiments performed with cells from at least 2 to 5 different donors. The amount of unscheduled DNA synthesis in control cells, measured as the incorporation of [^{3}H]dTMP, was 23 000–30 000 dpm $(10^6$ cells$)^{-1}$ $(6\ h)^{-1}$.

MNNG-treated cells incubated with 6-mercaptopurine, pyrazinamide, and theophylline. Theophylline retarded the rate of NAD^+ lowering more than pyrazinamide; pyrazinamide retarded the rate of lowering more than 6-mercaptopurine. The rate of NAD^+ lowering was even more retarded when cells were incubated with nicotinamide or benzamide. These compounds were the most potent inhibitors of polymerase activity. The capacity of these analogues to retard NAD^+ lowering decreased in the same order as their ability to inhibit poly(ADP-ribose) polymerase: benzamide > nicotinamide > theophylline > pyrazinamide > 6-mercaptopurine. Although these compounds retarded the rate of NAD^+ lowering, they did not prevent it. As shown in Figure 1, the NAD^+ pool in

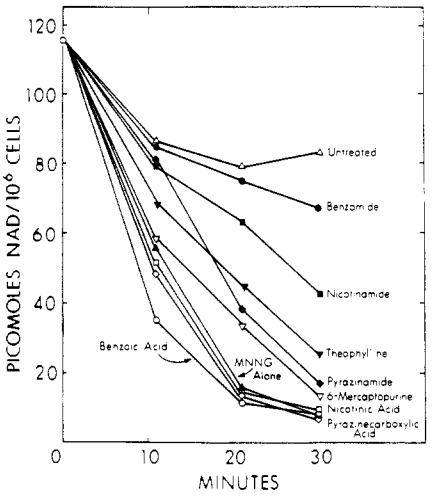

FIGURE 3: Effect of nicotinamide and purine analogues on NAD^+ lowering caused by MNNG treatment. Symbols: resting lymphocytes were either untreated (△) or treated with 136 μM MNNG alone (▲). All other cells were treated with 136 μM MNNG plus the following inhibitors: 2 mM benzamide (●); 2 mM nicotinamide (■); 2 mM theophylline (▼); 2 mM pyrazinamide (♦); 2 mM 6-mercaptopurine (▽); 2 mM nicotinic acid (□); 2 mM pyrazinecarboxylic acid (◇); 2 mM benzoic acid (○).

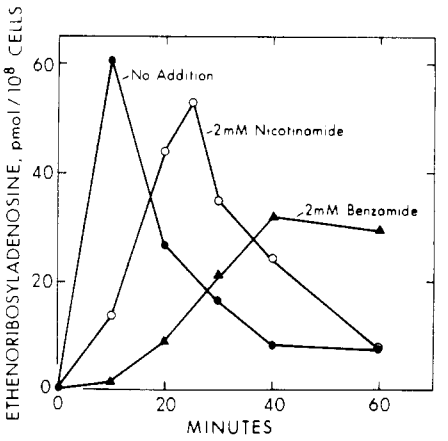

FIGURE 4: Poly(ADP-ribose) levels in MNNG-treated resting lymphocytes. At the indicated time after MNNG treatment, samples were removed from cell cultures, and poly(ADP-ribose) was extracted and analyzed as described under Methods. Symbols: resting lymphocytes treated with 136 μM MNNG and no further addition (●), 136 μM MNNG and 2 mM nicotinamide (○), or 136 μM MNNG and 2 mM benzamide (▲).

UV-irradiated cells is lowered after 2 h even in the presence of 2 mM nicotinamide. We have also measured NAD^+ levels at hourly intervals for 6 h after MNNG treatment; within 2 h there is little or no difference in NAD^+ levels in cells incubated in medium or in medium containing 2 mM nicotinamide. Thus, while there was a good correlation between polymerase inhibition in vitro and reduced rates of NAD^+ lowering in vivo, even the most effective polymerase inhibitors could not prevent the lowering of NAD^+ levels.

To determine whether these analogues actually affect the level of polymer synthesis in vivo, we measured poly(ADP-ribose) levels in MNNG-treated cells incubated in the presence of analogues with varying capacities to inhibit the polymerase. Poly(ADP-ribose) levels in resting lymphocytes from nine donors varied between undetectably low levels to 3 pmol of monomeric units measured as ethenoribosyladenosine/10^8 cells. Figure 4 shows that within 10 min after treatment of the cells with MNNG there was an increase in poly(ADP-ribose) such that the levels in MNNG-treated cells were 60–70 pmol of

Table III: Effect of Inhibitors on Poly(ADP-ribose) Levels in MNNG-Treated Human Lymphocytes

treatment [a]	poly(ADP-ribose) levels [b]	% inhibn
control	0–3	
136 μM MNNG	60	
136 μM MNNG + benzoic acid	72	(22% stim)
136 μM MNNG + nicotinic acid	47	22
136 μM MNNG + pyrazinecarboxylic acid	64	(6% stim)
136 μM MNNG + benzamide	1	98
136 μM MNNG + nicotinamide	16	73
136 μM MNNG + pyrazinamide	21	66

[a] Analogues were present at a concentration of 2 mM. Me_2SO was present in the control and in all MNNG-treated cells at a final concentration of 0.2%. [b] Picomoles of ethenoribosyladenosine per 10^8 cells. Polymer levels were measured at 10 min after indicated treatments.

ethenoribosyladenosine/10^8 cells. Similar measurements in cells derived from four different donors showed that peak levels of poly(ADP-ribose) were always obtained within 10–15 min of MNNG treatment. Polymer levels decreased rapidly and returned to less than 8 pmol of ethenoribosyladenosine/10^8 cells by 40 min. Thus, poly(ADP-ribose) has a very short half-life in vivo in MNNG-treated cells. When cells were treated with MNNG and immediately incubated with inhibitors of polymerase activity such as benzamide, nicotinamide, and pyrazinamide, they had greatly reduced polymer levels at 10 min as shown in Table III. When cells were incubated with analogues that did not inhibit polymerase activity such as benzoic acid, nicotinic acid, or pyrazinecarboxylic acid, polymer levels were not significantly reduced as compared to cells treated with MNNG alone. In fact, some stimulation of polymer levels occurred in benzoic acid and in pyrazinecarboxylic acid treated cells.

We subsequently examined the time course of polymer synthesis in MNNG-treated cells incubated in the presence and absence of nicotinamide and benzamide. The results of these studies are shown in Figure 4. Treatment with MNNG alone caused polymer levels to rise and reach a maximum at 10 min. MNNG-treated cells, incubated in the presence of 2 mM nicotinamide, showed a delay in the increase in polymer levels such that the maximum level was attained at 20–30 min after treatment with MNNG. There was never more polymer in nicotinamide-treated cells than at the peak of synthesis in control cells. MNNG-treated cells incubated in the presence of 2 mM benzamide showed an even greater lag before polymer levels began to increase. Polymer levels rose slowly to a maximum of 32 pmol of ethenoribosyladenosine/10^8 cells in 40 min and remained relatively constant through the 1-h time period examined. Benzamide-treated cells always had lower polymer levels than those reached at 10 min in cells treated with MNNG alone. The nicotinamide and benzamide treatments caused the cells to have persistently higher polymer levels at later times than were present in cells incubated in the absence of these compounds. The delay in the polymer level increase closely followed the ability of nicotinamide and benzamide to inhibit the polymerase in vitro. Benzamide was a more effective inhibitor than was nicotinamide and benzamide more effectively retarded the increase in polymer levels than did nicotinamide. Thus, the polymerase inhibitors do not prevent the lowering of NAD^+ or the synthesis of poly(ADP-ribose), but rather they slow the rate of NAD^+ lowering and retard the rate of poly(ADP-ribose) accumulation.

Stimulation of DNA Repair Synthesis. For evaluation of the ability of the analogues to stimulate unscheduled DNA synthesis, resting lymphocytes were UV irradiated or treated with MNNG and incubated with 10 mM hydroxyurea in the presence or absence of different analogues. The amount of [^{3}H]dTMP incorporated by DNA-damaged cells incubated in the presence of hydroxyurea was taken as the 100% level for unscheduled DNA synthesis. Stimulation of unscheduled DNA synthesis in the presence of the analogues was expressed relative to the 100% level. The fourth column in Table II shows the effect of the analogues on unscheduled DNA synthesis in UV-irradiated lymphocytes. The ability to stimulate unscheduled DNA synthesis showed a good correlation with the ability to inhibit poly(ADP-ribose) polymerase. Agents such as benzamide (compound **18**), 3-aminobenzamide (compound **19**), 3-methoxybenzamide (compound **20**), nicotinamide (compound **1**), picolinamide (compound **2**), and theobromine (compound **30**) which were the best polymerase inhibitors showed the greatest capacity to stimulate unscheduled DNA synthesis. Analogues such as 2-aminobenzamide (compound **21**), 4-aminobenzamide (compound **22**), pyrazinamide (compound **25**), 6-aminonicotinamide (compound **4**), isonicotinamide (compound **5**), 3-acetylpyridine (compound **7**), and 6-mercaptopurine (compound **33**), which were intermediate in their ability to inhibit polymerase activity, were intermediate in their ability to stimulate unscheduled DNA synthesis. Those compounds which were unable to inhibit polymerase activity showed no ability to stimulate unscheduled DNA synthesis. It was particularly striking to find that the carboxylic acid compounds, such as nicotinic acid (compound **12**), picolinic acid (compound **13**), and pyrazinecarboxylic acid (compound **27**), were unable to stimulate unscheduled DNA synthesis while the carboxamide compounds nicotinamide (compound **1**), picolinamide (compound **2**), and pyrazinamide (compound **25**) stimulated unscheduled DNA synthesis. Compounds containing a modified carboxamide group, for example, *N*-methylnicotinamide (compound **10**) and diethylnicotinamide (compound **11**), were unable to stimulate unscheduled DNA synthesis. Thus, the carboxamide group is required for stimulation of unscheduled DNA synthesis just as it is required for polymerase inhibition. It was also interesting to find that among the strongest inhibitors of the polymerase the ability to stimulate unscheduled DNA synthesis followed the potency of polymerase inhibition. For example, 5-methylnicotinamide (compound **3**) stimulated unscheduled DNA synthesis 175% and inhibited the polymerase 83%; 3-aminobenzamide (compound **19**) stimulated unscheduled DNA synthesis 215% and inhibited the polymerase 96%. Thus, the structural requirements for the stimulation of unscheduled DNA synthesis are the same as those for inhibition of poly(ADP-ribose) polymerase. Furthermore, the most potent stimulators of unscheduled DNA synthesis were also the most potent inhibitors of the poly(ADP-ribose) polymerase, the most effective agents in retarding the rate of NAD^+ lowering, and the most effective in delaying the accumulation of poly(ADP-ribose).

With the exception of nicotinamide itself, none of the nicotinamide, benzamide, pyrazinamide, and purine analogues can be incorporated into a true NAD^+ molecule. These studies show that the stimulation of unscheduled DNA synthesis is not mediated by the synthesis of NAD^+. However, it is possible that some of the analogues of the pyridine, pyrazine, and purine series might be utilized for the synthesis of NAD^+

analogues. Since the benzamides lack a ring nitrogen, they should not be able to replace nicotinamide for the synthesis of an NAD^+ analogue. Thus, the benzamides provide the best evidence that the ability to stimulate unscheduled DNA synthesis is correlated with the ability to inhibit poly(ADP-ribose) polymerase in vivo rather than the ability to stimulate NAD^+ synthesis.

Discussion

We have previously shown that nicotinamide stimulates unscheduled DNA synthesis in resting lymphocytes following DNA-damaging treatments (Berger & Sikorski, 1980). Using bromodeoxyuridine incorporation and density shift experiments, we have also shown that the unscheduled DNA synthesis that occurs in human lymphocytes in response to treatment with DNA-damaging agents represents the repair mode of DNA synthesis (Berger et al., 1979). Most of the present measurements of unscheduled DNA synthesis were conducted during a 6-h incubation with [^{3}H]thymidine in the presence of 10 mM hydroxyurea. As shown in Table I, for our studies, the presence or absence of 10 mM hydroxyurea had negligible effects on the ability of nicotinamide to stimulate unscheduled DNA synthesis. We also found that hydroxyurea had no effects on the ability of the other nicotinamide analogues to stimulate unscheduled DNA synthesis in DNA-damaged cells. In addition, we have previously used a permeable cell system in which no hydroxyurea was present to demonstrate that nicotinamide stimulates unscheduled DNA synthesis in DNA-damaged cells (Berger et al., 1979). Furthermore, Oikawa et al. (1980) have shown that several of these nicotinamide analogues stimulate sister chromatid exchange in DNA-damaged cells incubated in the absence of hydroxyurea. More recently, Durkacz et al. (1981) treated L1210 cells with dimethyl sulfate, then incubated them in 2.5 mM hydroxyurea and bromodeoxyuridine, and then analyzed DNA repair synthesis by alkaline cesium chloride gradients. Using this technique, they showed that nicotinamide analogues enhanced incorporation into DNA repair patches. Thus, the ability of nicotinamide and its analogues to enhance unscheduled DNA synthesis and DNA repair has now been shown in the presence and absence of a variety of concentrations of hydroxyurea, in whole cell studies, in permeable cell preparations, and by alkaline cesium chloride gradient techniques.

In the process of evaluating the mechanism by which nicotinamide stimulates DNA repair synthesis, we have recently shown that it does not exert its effect through the induction of polyamine or protein synthesis (Sims et al., 1981). Since nicotinamide is a precursor for NAD^+ biosynthesis and also a poly(ADP-ribose) polymerase inhibitor, we have examined the effect of other poly(ADP-ribose) polymerase inhibitors on the process of unscheduled DNA synthesis to determine which of the potential nicotinamide functions best correlates with the ability to stimulate unscheduled DNA synthesis. These studies show that DNA damage caused by UV irradiation or MNNG causes acute lowering of intracellular NAD^+ levels, such that within 1 h the NAD^+ levels are less than 10% of controls. This is in agreement with the studies of Rankin et al. (1980), who showed that DNA damage produced by MNNG, methyl methanesulfonate, and AAAF caused acute lowering of lymphocyte NAD levels. We have now shown that MNNG treatment of normal human lymphocytes stimulates the rapid synthesis of poly(ADP-ribose), and this synthesis is apparently responsible for the abrupt NAD^+ lowering. Similar kinetics of NAD^+ lowering and poly(ADP-ribose) synthesis have also been observed in 3T3 cells treated with MNNG (Jaurez-Salinas et al., 1979).

In the present study we used a permeable cell assay to identify a series of analogues that were highly effective inhibitors of poly(ADP-ribose) polymerase. When these analogues were tested in intact cells, we found that the rapid lowering of NAD^+ induced by MNNG treatment was greatly reduced. However, even the most effective inhibitors could not prevent the lowering of NAD^+; they only retarded its rate of fall. Similarly, the analogues which inhibited the polymerase in permeable cells reduced, but did not prevent, the increase in intracellular polymer levels that occurred in MNNG-treated cells. In contrast, NAD^+ lowering was unaffected by analogues which did not inhibit the polymerase in the permeable cell assay. Likewise, the increase in intracellular poly(ADP-ribose) levels measured at 10 min was not inhibited by incubation of MNNG-treated cells with analogues which did not inhibit poly(ADP-ribose) polymerase in permeable cell assays. These studies clearly indicate that the ability of nicotinamide and other analogues of NAD^+ to stimulate DNA repair synthesis in normal human lymphocytes correlates with their ability to inhibit poly(ADP-ribose) polymerase, their ability to reduce the rate of NAD^+ lowering, and their ability to delay the increase in intracellular poly(ADP-ribose) levels that occurs after DNA-damaging treatments. While these correlations are quite strong, there is no direct proof that the ability of the analogues to stimulate unscheduled DNA synthesis is dependent on their ability to inhibit poly(ADP-ribose) polymerase. Since the same analogues are potential inhibitors of the enzymes responsible for mono-ADP-ribosylations, it is possible that such enzymes represent the mechanism through which unscheduled DNA synthesis is stimulated. It is also possible that the effect of these agents are mediated through an effect on the chain length of poly(ADP-ribose) synthesized. We are currently developing methods to measure these parameters and their response to these analogues.

Our results showing that NAD^+ analogues stimulate unscheduled DNA synthesis are somewhat surprising since previous studies have shown that inhibitors of poly(ADP-ribose) synthesis interfere with both the DNA repair process and the ability of L1210 cells to survive after treatment with the DNA-damaging agent, methyl methanesulfonate (Durkacz et al., 1980). Our observations that nicotinamide and its analogues stimulate unscheduled DNA synthesis are not confined to the resting human lymphocyte since Althaus et al. (1980) have shown that nicotinamide and isonicotinamide stimulate unscheduled DNA synthesis in primary rat hepatocyte cultures and we as well as Durkacz et al. (1981) have recently found similar results in L1210 cells treated with nicotinamide and other analogues.

It has previously been suggested that the synthesis of poly(ADP-ribose) that occurs in response to DNA damage serves to modify chromosomal proteins and conformation so that the enzymes of DNA repair can gain access to regions of damaged DNA (Butt et al., 1978; Berger et al., 1979; Durkacz et al., 1980). It is possible that under the conditions of DNA damage used in these studies, there is an overstimulation of poly(ADP-ribose) polymerase, leading to a level of chromatin ADP-ribosylation and modification that interferes with the efficient progress of the DNA repair process. The ability of the inhibitors to slow down the synthesis of poly(ADP-ribose) might allow for a more efficient DNA repair process and subsequent restitution of chromatin structure. Another possibility which might explain the effect of nicotinamide and the other NAD^+ analogues on the DNA repair process relates to their effects on the NAD^+ pool size. The

stimulation of poly(ADP-ribose) by UV irradiation or MNNG treatment results in a rapid depletion of cellular NAD^+ levels which may render the cells unable to maintain their redox state and energy metabolism. Thus, the rapid depletion of NAD^+ may lead to a metabolic cell death before the DNA repair processes can be completed. The effect of nicotinamide and the NAD^+ analogues could be to slow down the reduction in NAD^+ levels so that the cells maintain sufficient pyridine nucleotide coenzymes to continue energy metabolism and repair DNA under more controlled conditions.

References

Althaus, F. R., Lawrence, S. D., Sattler, G. L., & Pitot, H. C. (1980) *Biochem. Biophys. Res. Commun. 95*, 1063–100.

Benjamin, R. C., & Gill, D. M. (1980) *J. Biol. Chem. 225*, 10493–10501.

Berger, N. A., & Sikorski, G. W. (1980) *Biochem. Biophys. Res. Commun. 95*, 67–72.

Berger, N. A., Sikorski, G. W., & Petzold, S. J., & Kurohara, K. K. (1979) *J. Clin. Invest. 63*, 1164–1171.

Butt, T. F., Brothers, J. F., Giri, C. P., & Smulson, M. E. (1978) *Nucleic Acids Res. 5*, 2775–2788.

Collins, A. R. S., Schor, S. L., & Johnson, R. T. (1977) *Mutat. Res. 42*, 413–432.

Cornelis, J. J. (1978) *Biochim. Biophys. Acta 521*, 134–143.

Durkacz, B. W., Omidiji, O., Gray, D. A., & Shall, S. (1980) *Nature (London) 283*, 593–596.

Durkacz, B. W., Irwin, J., & Shall, S. (1981) *Biochem. Biophys. Res. Commun. 101*, 1433–1441.

Francis, A. A., Blevins, R. D., Carrier, W. L., Smith, D. P., & Regan, J. D. (1979) *Biochim. Biophys. Acta 563*, 385–392.

Goodwin, P. M., Lewis, P. J., Davis, M. I., Skidmore, C. J., & Shall, S. (1978) *Biochim. Biophys. Acta 543*, 576–582.

Hayaishi, O., & Ueda, K. (1977) *Annu. Rev. Biochem. 46*, 95–116.

Hilz, H., & Stone, P. (1976) *Rev. Physiol., Biochem. Pharmacol. 76*, 1–58.

Jacobson, E. L., Juarez, D., & Sims, J. L. (1980) *Fed. Proc., Fed Am. Soc. Exp. Biol. 39 (No. 6)*, 1739A.

Jacobson, M. K., Levi, V., Jaurez-Salinas, H., Barton, R. A., & Jacobson, E. L. (1980) *Cancer Res. 40*, 1797–1802.

Jaurez-Salinas, H., Sims, J. L., & Jacobson, M. K. (1979) *Nature (London) 282*, 740–741.

Kato, T., Berger, S. J., Carter, J. A., & Lowry, O. H. (1973) *Anal. Biochem. 53*, 86–97.

Mendelsohn, J. S., Skinner, S. A., & Kornfeld, S. (1971) *J. Clin. Invest. 50*, 818–826.

Miller, E. G. (1977) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 36 (No. 3)*, 3349A.

Oikawa, A., Tohda, H., Kanai, M., Miwa, M., & Sugimura, T. (1980) *Biochem. Biophys. Res. Commun. 97*, 1311–1316.

Petzold, S. J., Booth, B. A., Leimback, G. A., & Berger, N. A. (1981) *Biochemistry 20*, 7075–7081.

Rankin, P. W., Jacobson, M. K., Mitchell, V. R., & Busbee, D. L. (1980) *Cancer Res. 40*, 1803–1807.

Sims, J. L., Jaurez-Salinas, H., & Jacobson, M. K. (1980) *Anal. Biochem. 106*, 296–306.

Sims, J. L., Berger, S. J., & Berger, N. A. (1981) *J. Supramol. Struct. Cell. Biochem. 16*, 281–288.

Steady-State Fluorescence Polarization of Dansylcadaverine–Fibrinogen: Evidence for Flexibility†

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ABSTRACT: The conformation of fibrinogen in solution has been investigated by steady-state fluorescence polarization measurements. Factor XIIIa has been employed to enzymatically incorporate 1–6 mol of dansylcadaverine/mol of fibrinogen into a specific glutamine residue near the carboxy terminus of the γ chain and up to two sites on the α chain. The fluorescence emission maximum of the labeled protein is shifted to 495 nm (from 538 nm for the fluorophore in solution) and the intensity substantially enhanced, indicating the covalently linked dansyl groups residue in a hydrophobic environment in the interior of the protein. This covalent modification does not interfere with the formation of fibrin, following thrombin activation. Steady-state fluorescence polarization measurements were carried out as a function of temperature and in high viscosity solvents. The fluorescent lifetime of dansylcadaverine–fibrinogen was determined by a phase shift technique. Analysis of the data by the Perrin–Weber treatment yields a rotational relaxation time of 160 ns, considerably faster than any realistic hydrodynamic model of fibrinogen would predict. The results are discussed in terms of segmental flexibility.

Fibrinogen is a soluble plasma protein of molecular weight 340 000 comprised of three pairs of polypeptide chains ($A\alpha$, $B\beta$, and γ) which are covalently linked by numerous disulfide bonds. Although the primary structure of fibrinogen has been determined (Henschen & Lottspeich, 1980; Doolittle, 1980), molecular details of its three-dimensional structure are still under active investigation. Hall & Slayter (1959) proposed a trinodular structure for fibrinogen, based on electron micrographs of shadowed specimens, in which two outer spheres 6–7 nm in diameter are connected by thin threads to a smaller central nodule (5-nm diameter). Hydrodynamic data indicate that fibrinogen can be treated as a long prolate ellipsoid, although uncertainties concerning the degree of hydration and contributions of the less ordered portions of the α chains

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