PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 183, 221–226 (1986)

Increased Ionic Strength: Effects on DNA Fragmentation and Poly(ADP-Ribose) Polymerase Activity in HeLa Nuclei (42408)

EDWARD G. MILLER

Department of Biochemistry, Baylor College of Dentistry, Dallas, Texas 75246

Abstract. Poly(ADP-ribose) polymerase activity was measured in a crude nuclear fraction isolated from HeLa cells. It was found that the addition of ammonium sulfate or other salts to the standard incubation medium inhibited the formation of poly(ADP-ribose). Through the use of alkaline sucrose density gradients it was also noted that this same increase in ionic strength inhibited the *in vitro* breakdown of the HeLa DNA. Additional experiments with alkaline sucrose density gradients and deoxyribonuclease I showed that the *in vitro* activity of poly(ADP-ribose) polymerase is largely dependent upon DNA fragmentation but that DNA fragmentation at least *in vitro* is not dependent upon the formation of poly(ADP-ribose). These observations imply that this nuclear enzyme is not extremely sensitive to changes in the ionic strength of the reaction media but is affected indirectly, supposedly through changes in the endonuclease activity of the HeLa nuclei. If this proves to be true, then the addition of salt to the incubation medium for poly(ADP-ribose) polymerase could prove to be a valuable tool for the study of ADP-ribosylation reactions.

Previous research (1, 2) in this laboratory has concentrated on the effect of exogenous endonucleases on the poly(ADP-ribose) polymerase activity of HeLa nuclei. It has been shown that the addition of either DNAase I or micrococcal nuclease to the complete reaction mixture can increase by four- to sixfold the incorporation of NAD into an acid-precipitable product. Additional studies with DNAase I have shown that this product is poly(ADP-ribose) and that this increase in activity is due to an increase in the synthesis of new chains of poly(ADP-ribose).

In many ways these investigations were a comparison of two assay conditions, one with DNAase I and the other without the added endonuclease. In almost every instance the two assays responded in the same way to additional changes in the incubation conditions; however, there was one exception. The addition of ammonium sulfate to the two media produced a differential effect. It was found that the assay without DNAase I was readily inhibited by increasing the concentration of ammonium sulfate in the incubation mixture. This result agrees with the findings of other investigators (3, 4). However, in the assay with DNAase I the inhibition by ammonium sulfate was considerably smaller.

This difference in response in these two assays has led us to a reexamination of the effect of different salts on the activity of poly(ADP-ribose) polymerase. The results of this study are presented in this paper and indicate a complex relationship between the poly(ADP-ribose) polymerase activity and the endogenous endonuclease activity of HeLa nuclei. The data show that this relationship can be partially disrupted by raising the ionic strength of the incubation medium. The data also indicate that this effect can be utilized to increase the sensitivity of *in vitro* assays for poly(ADP-ribose) polymerase.

Materials and Methods. Unlabeled NAD was purchased from Sigma (St. Louis, Mo.), while DNAase I (electrophoretically purified) was obtained from Worthington (Freehold, N.J.). Tritiated thymidine and [^{3}H]NAD were purchased from New England Nuclear (Boston, Mass.).

HeLa cells were maintained in monolayers as previously described (1). The cells, bottles, media, and supplements were purchased from Microbiological Associates (Bethesda, Md.). When the cells reached confluence, the monolayers were trypsinized and a crude nuclear fraction was prepared (1). The assay conditions for the measurement of poly(ADP-ribose) polymerase activity were described in an earlier publication (1).

Density gradients were used to measure the effect of different incubation conditions and

0037-9727/86 $1.50

media on the integrity of the nuclear DNA. For these experiments the cells were prelabeled in culture with tritiated thymidine (0.6 μCi/ml) for 48 hr. Following the labeling period the cells were harvested, and the nuclei were isolated and incubated. The different assay mixtures will be described in the text. After the incubation a portion of the medium was diluted 50-fold with a buffer containing 24 m*M* EDTA and 75 m*M* NaCl (pH 7.5). The diluted samples were immediately layered onto alkaline sucrose gradients that had been prepared in cellulose nitrate tubes. The composition of each gradient from the bottom to the top was 1 ml of 2.3 *M* sucrose, 10 ml of a linear sucrose gradient (5–20%) containing 0.9 *M* NaCl and 0.3 *M* NaOH, 0.25 ml of lysis solution (0.3 *M* NaCl, 30 m*M* EDTA, 0.5% sodium dodecyl sulfate, and 100 m*M* Tris–HCl, pH 10), 0.25 ml of the diluted nuclei (approximately 1 μg of DNA), another 0.1 ml of lysis solution, and mineral oil. The gradients were centrifuged in a SW 27.1 rotor in a Beckman L3-50 ultracentrifuge at 81,800*g* for 90 min at 22°C. After centrifugation the tubes were pierced and 15-drop fractions were collected in scintillation vials. The samples were diluted with distilled water, suspended in 10 ml of Scintisol, and counted in a liquid scintillation spectrometer. This technique is similar to the procedure of Cox *et al.* (5).

Results. Figure 1 illustrates the effect of ammonium sulfate on the poly(ADP-ribose) polymerase activity of HeLa nuclei. For this experiment the activity of the enzyme was measured in two different assay mixtures (± 25 μg of DNAase I). Earlier reports (1, 2, 6) have shown that DNAase I can stimulate by several-fold the poly(ADP-ribose) polymerase activity of isolated nuclei. From the graphs it is obvious that as the concentration of ammonium sulfate was increased the activity of the enzyme decreased; however, the overall inhibition was considerably more pronounced in the assay without DNAase I. At a concentration of 50 m*M* ammonium sulfate over 70% of the activity in the assay without DNAase I was lost; however, over 70% of the activity was still present in the assay with DNAase I. At a concentration of 200 m*M* ammonium sulfate, the activity in the assay without DNAase I was essentially nil (3%), but 40% of the activity remained in the assay with DNAase I. This

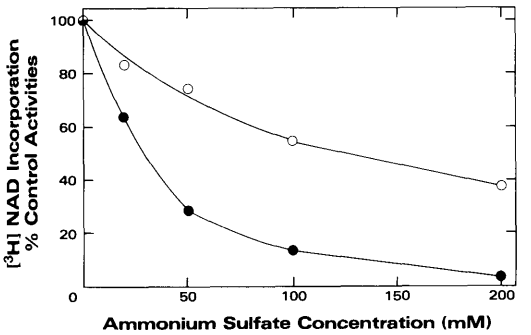

FIG. 1. The effect of ammonium sulfate on NAD incorporation. The concentration of ammonium sulfate was varied as shown. The incubation mixture contained either 25 μg of DNAase I (○) or no DNAase I (●). The average values (each assay was performed in duplicate) for the control tubes (no ammonium sulfate) were 20.6 nmole NAD without DNAase I and 88.5 nmole NAD with 25 μg of DNAase I. The remaining assay conditions were described in an earlier publication (1).

difference between the two assays was also seen with a wide variety of other salts (K_2SO_4, $NH_4C_2H_3O_2$, NaCl, CsCl, Cs_2SO_4, Li_2SO_4, NH_4Cl, and Na_2SO_4).

These results indicated that the two assays have different sensitivities to an increase in ionic strength; however, this differential effect on two systems both known to be synthesizing poly(ADP-ribose) was unexpected. Why would an increase in ionic strength essentially knock out one assay but have only a limited affect on the other? One possible explanation might be a difference in the turnover not the synthesis of poly(ADP-ribose). Since it has already been suggested (7) that the activity of one of the degradative enzymes, poly(ADP-ribose) glycohydrolase, is influenced by changes in the ionic strength of the incubation media, an experiment was performed to test this possibility.

For this experiment the nuclei were placed in the standard incubation media and allowed to synthesize labeled poly(ADP-ribose). After the primary incubation the nuclei were centrifuged, washed, and resuspended in a second assay mixture. This medium was similar to the medium that was used in the initial incubation. The only differences were the addition of 5 m*M* nicotinamide and the exclusion of NAD. Both of these changes were made to block the activity of poly(ADP-ribose)

polymerase. The tubes were incubated at 37°C and samples were taken at 15, 30, 60, and 120 min. The results (data not given) showed that the addition of 50 m*M* ammonium sulfate to this second reaction mixture did not affect the loss of radioactivity from the prelabeled nuclei. At the end of the 2-hr incubation 83% of the radioactivity remained in the assay with ammonium sulfate; 82% remained in the assay without ammonium sulfate.

Another possible explanation for the data in Fig. 1 is that the main effect of increased ionic strength at least in the assay without DNAase I is on an activator of poly(ADP-ribose) polymerase and considering our earlier work (1, 2) a potential candidate for this activator was felt to be one or more of the DNA-specific endonucleases of HeLa nuclei (8). This line of reasoning suggested two things. First, an increase in ionic strength should have little if any effect on the degradative activity of DNAase I in the DNAase I assay. Second, if an increase in ionic strength can inhibit one or more of the endogenous endonucleases, then there should be some difference in the size of the HeLa DNA after incubations under these two new assay conditions, one without salt and one with salt.

The data in Table I and Fig. 2 supports these predictions. From the table it can be seen that DNAase I can be used to increase the poly(ADP-ribose) polymerase activity of HeLa nuclei. It is also obvious that the complete breakdown of the nuclear DNA is not a prerequisite for this stimulatory effect. With the addition of 1 μg of DNAase I to the complete reaction mixture, the poly(ADP-ribose) polymerase activity of the HeLa nuclei was increased by 250%, but less than 5% of the DNA was rendered acid soluble. The last two lines in Table I show that the addition of 100 m*M* ammonium sulfate to the incubation mixture cannot block or inhibit to any significant extent the degradative activity of 25 μg of DNAase I. This result indicates that the decrease in poly(ADP-ribose) polymerase activity that was seen in the assay with DNAase I is probably due to a direct effect by the salt on the activity of poly(ADP-ribose) polymerase.

TABLE I. THE EFFECT OF DNAase I AND AMMONIUM SULFATE ON NAD INCORPORATION AND DNA DEGRADATION

Additions to assay	[^{3}H]NAD incorporation (nmole)	DNA[a] (μg)
None	13.1	61.8
1 μg DNAase I	44.4	58.8
5 μg DNAase I	77.4	16.7
25 μg DNAase I	91.0	0.9
25 μg DNAase I + 100 mM $(NH_4)_2SO_4$	56.3	2.3

[a] The diphenylamine assay (9) was used for the DNA determinations. The remaining experimental details were described in Fig. 1.

The data in Fig. 2 indicate that an increase in ionic strength can affect DNA fragmentation in the assay without DNAase I. The results show obvious differences in the profiles for the nuclear DNA after incubations either with or without 50 m*M* ammonium sulfate. With ammonium sulfate in the reaction mixture the profile for the HeLa DNA was shifted to the heavier side of the gradient and exhibited a narrower band of radioactivity. These changes demonstrate a protective effect by 50 m*M* ammonium sulfate on the HeLa DNA and suggest that an increase in ionic strength may be partially blocking the activity of one or more of the DNA-specific endonucleases of HeLa nuclei.

The latter possibility is supported by an experiment (data not shown) with nuclei incubated at 4°C instead of 37°C. These nuclei were incubated for 30 min in the medium without ammonium sulfate. After the incubation the nuclei were analyzed on an identical alkaline sucrose density gradient. The results showed a sharp peak of radioactivity near the bottom of the gradient (fractions 3 and 4). These data indicate that the fragmentation that is seen in Fig. 2 is temperature dependent and probably enzymatic in origin, and that 50 m*M* ammonium sulfate can only partially protect the DNA during an incubation at 37°C.

All of the results with ammonium sulfate and other salts indicate that an increase in ionic strength can inhibit the activity of poly(ADP-ribose) polymerase and can also inhibit the activity of one or more of the HeLa endonucleases. From our earlier data (1, 2) we assumed that the inhibition of the HeLa endonuclease(s) contributes to the inhibition of poly(ADP-ribose) polymerase. Evidence from other laboratories (10–12) has suggested that

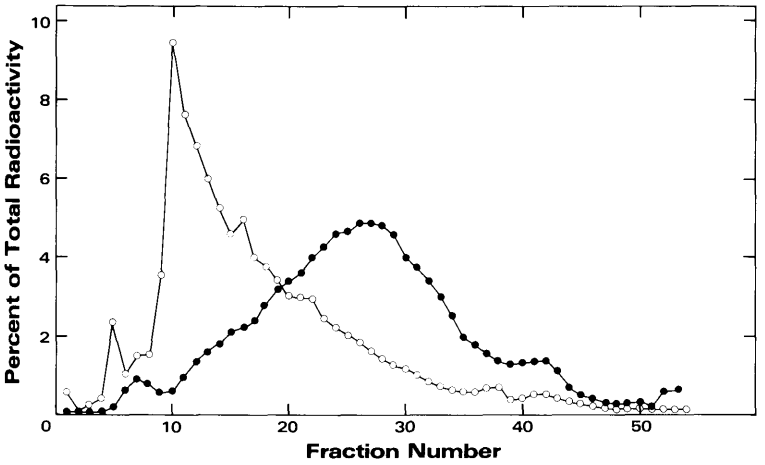

FIG. 2. The effect of ammonium sulfate and NAD on the sedimentation of nuclear DNA in alkaline sucrose gradients. For this experiment the HeLa DNA was labeled *in vivo* with radioactive thymidine. The nuclei were isolated and incubated for 30 min at 37°C. The incubation mixture (0.25 ml) contained 100 m*M* Tris–HCl, pH 8.0, 20 m*M* $MgCl_2$, 2.5 m*M* NAD, 0.4 m*M* β-mercaptoethanol, 0.2 m*M* EDTA, and either 50 m*M* $(NH_4)_2SO_4$ (○) or no $(NH_4)_2SO_4$ (●). This is similar to the media described in an earlier publication (1). The only difference is that [^{3}H]NAD and DNAase I have been excluded from the reaction mixture. The procedure for alkaline sucrose gradient centrifugation was described in the text. The recovery of radioactivity for both gradients was 100%. The sedimentation was from right (top) to left (bottom).

the opposite is true, that an inhibition of poly(ADP-ribose) polymerase activity will lead at least in certain instances to an inhibition of endonuclease activity. Both of these hypotheses can be used to explain the data in Fig. 2. A direct way to differentiate between them is shown in Fig. 3. If the synthesis of poly(ADP-ribose) is a prerequisite for endo-

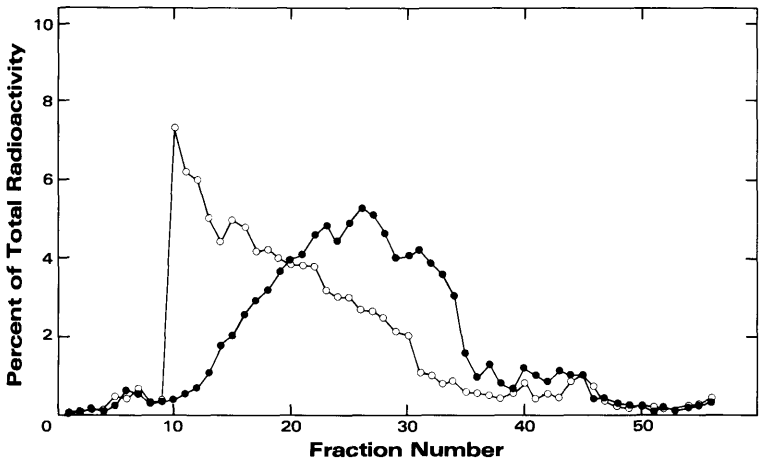

FIG. 3. The effect of ammonium sulfate on the sedimentation of nuclear DNA in alkaline sucrose gradients. This experiment is similar to the experiment that is illustrated in Fig. 2. The only difference was that NAD both labeled and unlabeled was excluded from the incubation mixture. As before the incubation media either contained 50 m*M* $(NH_4)_2SO_4$ (○) or no $(NH_4)_2SO_4$ (●). All of the other experimental details were described in Fig. 2. The recovery of radioactivity for the two gradients was 100%. The sedimentation was from right (top) to left (bottom).

nuclease activity, the complete removal of NAD from the incubation mixture will eliminate poly(ADP-ribose) polymerase activity and should protect the nuclear DNA. The profiles in Fig. 3 show that this is not the case. The breakdown of the HeLa DNA was only inhibited in the medium with 50 m*M* ammonium sulfate. This fact is even more apparent when Figs. 2 and 3 are compared. The data in the two separate experiments are essentially the same.

Discussion. The results of this study have shown that an increase in ionic strength will inhibit the *in vitro* activity of poly(ADP-ribose) polymerase; however, the extent of this inhibition was considerably less than previously reported (3, 4). An apparent reason for these contradictory results is the additional observation that an increase in ionic strength can partially block the *in vitro* breakdown of the HeLa DNA. These data (Figs. 2, 3) suggest that a considerable portion of the *in vitro* activity of poly(ADP-ribose) polymerase is dependent upon DNA fragmentation. The data also indicate that this dependence is disrupted through an increase in ionic strength. Under these same assay conditions our results have shown that DNA fragmentation is not affected by changes in poly(ADP-ribose) polymerase activity.

The reason for the breakdown of the HeLa DNA during the incubation at 37°C is not known; however, the results suggest that it is probably due to the activity of the HeLa endonucleases. This idea is certainly supported by previous research with exogenous endonucleases (1, 6, 13), radiation (13–17), carcinogens (14, 15, 18–21), and other DNA-damaging agents (14, 22). In each case DNA fragmentation appeared to elicit a response by poly(ADP-ribose) polymerase. These studies and others (23–25) indicate that this nuclear enzyme has a role in some forms of DNA repair.

As indicated a considerable amount of research is being conducted on the effects of DNA-damaging agents on poly(ADP-ribose) polymerase activity. According to our observations the standard *in vitro* assay for measuring the activity of this enzyme is, to a large extent, just a reflection of *in vitro* damage to the DNA. The addition of 50 m*M* ammonium sulfate to the assay mixture reduces this dependence and should prove useful for magnifying the effects of DNA-damaging agents. This type of change increases the sensitivity of the assay and has already been utilized in studies with X rays and bleomycin (22). It also appears probable that this new assay can be extended to work with permeabilized cells. In experiments with L1210 cells (data not shown) permeabilized by the method of Berger *et al.* (26), the addition of 50 m*M* ammonium sulfate to the standard assay for poly(ADP-ribose) polymerase reduced the activity in this assay by 60–70%. In a similar assay with 25 μg of DNAase I, the addition of 50 m*M* ammonium sulfate to the reaction mixture only lowered the overall activity by 5–10%. These results are very similar to what was seen in HeLa nuclei (Fig. 1).

The author thanks Karla Roffee, S. Y. Huang, and J. C. Kenny, III, for their excellent technical assistance, and Rhonda Bryant for her help in the preparation of this manuscript. This investigation was supported by a grant from the Southwestern Medical Foundation from its Holley–Franklin Medical Research Trust.

1. Miller EG. Stimulation of nuclear poly(adenosine diphosphate-ribose) polymerase activity from HeLa cells by endonucleases. Biochim Biophys Acta **395**:191–200, 1975.
2. Miller EG. Effect of deoxyribonuclease I on the number and length of chains of poly(ADP-ribose) synthesized, in vitro. Biochem Biophys Res Commun **66**: 280–286, 1975.
3. Ueda K, Reeder RH, Honjo T, Nishizuka Y, Hayaishi O. Poly adenosine diphosphate ribose synthesis associated with chromatin. Biochem Biophys Res Commun **31**:379–385, 1968.
4. Stone PR, Shall S. Poly(adenosine diphosphoribose) polymerase in mammalian nuclei. Eur J Biochem **38**: 146–152, 1973.
5. Cox R, Damjanov I, Abanobi SE, Sarma DSR. A method for measuring DNA damage and repair in the liver in vivo. Cancer Res **33**:2114–2121, 1973.
6. Janakidevi K, Koh C. Synthesis of polyadenosine diphosphate ribose by isolated nuclei of swine aortic tissue. Biochemistry **13**:1327–1330, 1974.
7. Stone PR, Lorimer WS, Ranchalis J, Danley M, Kidwell WR. Effect of DNA on poly(ADP-ribose) glycohydrolase and the degradation of histone H1-poly(ADP-ribose) complex from HeLa cell nuclei. Nucleic Acid Res **5**:173–184, 1978.
8. Urbanczyk J, Studzinski GP. Chromatin-associated DNA endonuclease activities in HeLa cells. Biochem Biophys Res Commun **59**:616–622, 1974.
9. Burton KA. Conditions and mechanism of the di-

phenylamine reaction for the estimation of deoxyribonucleic acid. Biochem J **62**:315–323, 1956.
10. Roberts JH, Stark P, Smulson M. Poly(ADP-ribose): Release of template restriction in HeLa cells. Proc Natl Acad Sci USA **71**:3212–3216, 1974.
11. Smulson M, Stark P, Gazzoli M, Roberts J. Release of template restriction for DNA synthesis by poly ADP(ribose) polymerase during the HeLa cell cycle. Exp Cell Res **90**:175–182, 1975.
12. Wielckens K, George E, Pless T, Hilz H. Stimulation of poly(ADP-ribosyl)ation during Ehrlich ascites tumor cell "starvation" and suppression of concomitant DNA fragmentation by benzamide. J Biol Chem **258**: 4098–4104, 1983.
13. Benjamin RC, Gill DM. ADP-ribosylation in mammalian cell ghosts. J Biol Chem **255**:10493–10501, 1980.
14. Berger NA, Sikorski GW, Petzold SJ, Kurohara KK. Association of poly(adenosine diphosphoribose) synthesis with DNA damage and repair in normal human lymphocytes. J Clin Invest **63**:1164–1171, 1979.
15. Skidmore CJ, Davies MI, Goodwin PM, Halldorsson H, Lewis PJ, Shall S, Zia'ee AA. The involvement of poly(ADP-ribose) polymerase in the degradation of NAD caused by γ-radiation and N-methyl-N-nitrosourea. Eur J Biochem **101**:135–142, 1979.
16. Jacobson EL, Antol KM, Juarez-Salinas H, Jacobson MK. Poly(ADP-ribose) metabolism in ultraviolet irradiated human fibroblasts. J Biol Chem **258**:103–107, 1983.
17. Zwelling LA, Kerrigan D, Mattern MR. Ataxiatelangiectasia cells are not uniformly deficient in poly(ADP-ribose) synthesis following x-irradiation. Mutat Res **120**:69–78, 1983.
18. Sudhakar S, Tew KD, Schein PS, Woolley PV, Smulson ME. Nitrosourea interaction with chromatin and effect on poly(adenosine diphosphate ribose) polymerase activity. Cancer Res **39**:1411–1417, 1979.
19. Romaschin AD, Kirsten E, Jackowski G, Kun E. Quantitative isolation of oligo- and polyadenosine-diphosphoribosylated proteins by affinity chromatography from livers of normal and dimethylnitrosamine-treated Syrian hamsters. J Biol Chem **256**:7800–7805, 1981.
20. Wielckens K, Schmidt A, George E, Bredehorst R, Hilz H. DNA fragmentation and NAD depletion. J Biol Chem **257**:12872–12877, 1982.
21. Jacobson EL, Smith JY, Mingmuang M, Meadows R, Sims JL, Jacobson MK. Effect of nicotinamide analogues on recovery from DNA damage in C3H10T 1/2 cells. Cancer Res **44**:2485–2492, 1984.
22. Miller EG. Stimulation of poly(adenosine diphosphate-ribose) polymerase activity by bleomycin. Fed Proc **36**:906, 1977.
23. Creissen D, Shall S. Regulation of DNA ligase activity by poly(ADP-ribose). Nature (London) **296**:271–272, 1982.
24. Althaus FR, Lawrence SD, Sattler GL, Pitot HC. ADP-ribosyltransferase activity in cultured hepatocytes: Interactions with DNA repair. J Biol Chem **257**:5528–5535, 1982.
25. Cleaver JE, Bodell WJ, Morgan WF, Zelle B. Differences in the regulation by poly(ADP-ribose) of repair of DNA damage from alkylating agents and ultraviolet light according to cell type. J Biol Chem **258**:9059–9068, 1983.
26. Berger NA, Weber G, Kaichi AS. Relation of poly(adenosine diphosphoribose) synthesis to DNA synthesis and cell growth. Biochim Biophys Acta **519**: 87–104, 1978.

Received April 21, 1986. P.S.E.B.M. 1986, Vol. 183.
Accepted July 14, 1986.