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# Changes in Poly(adenosine diphosphate-ribose) and Poly(adenosine diphosphate-ribose) Polymerase in Synchronous HeLa Cells†

William R. Kidwell* and Michael G. Mage

ABSTRACT: An antibody has been prepared which is highly specific for poly(adenosine diphosphate-ribose). Neither poly(A), DNA, nor a variety of adenine-containing nucleosides or nucleotides were effective in competing with poly(ADP-ribose) for binding to the antibody. Of all compounds tested, only adenosine diphosphate-ribose competed for binding to the antibody. Unlabeled poly(adenosine diphosphate-ribose) was about 10 000 times more effective in competing with labeled polymer for antibody binding than was adenosine diphosphate-ribose. Using the antibody, the amount of poly(adenosine diphosphate-ribose) was found to increase from early S phase to a peak at mid S with a second, even larger increase seen at the S-$G_2$ transition point in synchronously dividing HeLa cells. Pulse labeling of the polymer with [2-$^3$H]adenosine was also maximal at the same time points. Changes in the levels of poly(adenosine diphosphate-ribose) polymerase activity measured in isolated nuclei coincided with the changes in amounts of polymer present in intact cells during progression from S phase into $G_2$.

The homopolymer, poly(ADP-ribose)[1] is synthesized from NAD by an enzyme, poly(ADP-ribose) polymerase, which is tightly associated with the chromatin of eukaryotic cells (Chambon et al., 1966; Hasegawa et al., 1967; Reeder et al., 1967). Although the function of the polymer is not known, the fact that it appears in part to be covalently joined to $f_1$ histones in vitro (Nishizuka et al., 1969) and in vivo (Ueda et al., 1975) may indicate that it plays an important role in modifying chromatin structure.

Two lines of evidence suggest that the polymer plays some role at or near the time of the S-$G_2$ transition point in

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[1] Abbreviations used: ADP-ribose, adenosine diphosphate-ribose; phosphoribosyl-AMP, 2′-(5″-phosphoribosyl)-5′-AMP; poly(ADP-ribose), poly(adenosine diphosphate-ribose), a polymer of ADP-ribose subunits joined in a 1′-2′ ribose-ribose linkage.

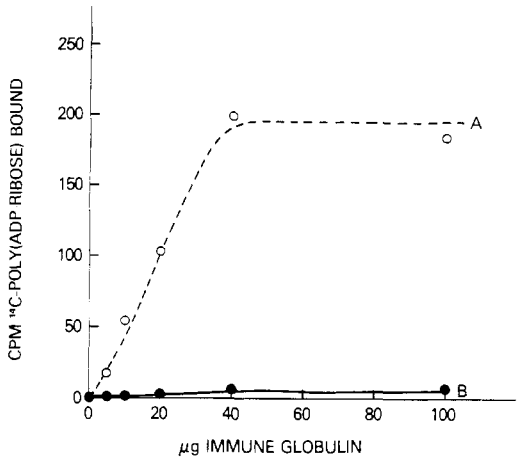


FIGURE 1: Detection of poly(ADP-ribose) binding antibody in the immune globulin fraction of rabbit serum. (A) Globulin fraction isolated 1 month after immunization. (B) Globulin fraction isolated from same animal before immunization. In the assay, 25 μl of [$^{14}$C]poly(ADP-ribose) (0.5 ng, 300 cpm) was added to 1 ml of 1 mM phosphate buffer, pH 6.8, containing various amounts of immune globulin protein. After incubation for 1 h at 37 °C, the samples were then directly filtered through Millipore filters (type HA), washed with 1 mM phosphate buffer, dried, and counted.

Table I: Effect of Various Adenine Nucleosides and Nucleotides on the Binding of Labeled Poly(ADP-ribose).[a]

| Additions | (cpm) Precipitation | % Inhibition |
|---|---|---|
| None | 620 | |
| 5′-ATP | 607 | 2.1 |
| 5′-ADP | 580 | 6.4 |
| 5′-AMP | 594 | 4.0 |
| Adenosine | 583 | 6.0 |
| 3′,5′-c-AMP | 578 | 6.7 |
| 2′,3′-AMP | 590 | 4.8 |
| ADP-ribose | 483 | 22.2 |
| Control serum[b] | 30 | |

[a] The assay was performed as described in Figure 1, except that immune serum was used rather than the purified immune globulin. After incubation and precipitation with cold 50% ammonium sulfate (final concentration), the radioactivity present in the pellet was extracted with hot acid and counted. Each assay tube contained 2 ng of labeled polymer and 50 μg of each nucleotide. [b] Equivalent amount of control serum added instead of immune serum.

growing cells. First, poly(ADP-ribose) polymerase activity peaks at this time in the cell cycle (Miwa et al., 1973; Kidwell and Watts, 1974) and there is a coincident maximum in the rate of incorporation of [$^3$H]adenosine into the polymer in intact cells (Kidwell and Watts, 1974). Second, an inhibitor of poly(ADP-ribose) polymerase, 5-methylnicotinamide, arrests the growth of HeLa cells between the end of the period of DNA synthesis and the time of mitosis (Kidwell and Burdette, 1974). The inhibitor may be specific since a variant HeLa cell has been isolated, the growth of which is not sensitive to the inhibitor. Furthermore, the polymerase activity in nuclei of the variant is less sensitive to inhibition by 5-methylnicotinamide than is that of the parental HeLa cell (Kidwell and Burdette, 1974).

Here we present an assessment of the changes in the mass of poly(ADP-ribose) in synchronous cells utilizing an immunoassay technique. Development of the assay was prompted by the finding that a specific antibody to the purified polymer can be produced in rabbits (Kanai et al., 1975).

## Materials and Methods

HeLa cells obtained from Flow Laboratories were maintained in shake cultures as previously described (Kidwell and Burdette, 1974). Periodic testing indicated that the cultures were free of mycoplasma contamination. Synchrony was achieved by exposure of cultures to hydroxyurea for 16 h, followed by replacement of the medium with fresh medium without hydroxyurea.

The poly(ADP-ribose) utilized for antiserum production was synthesized in calf thymus nuclei and purified through the hydroxylapatite step according to Miwa et al. (1971). Two further purification steps, necessary to remove residual contamination with protein and DNA, were as follows. Material eluting from hydroxylapatite between 0.35 and 1.0 M phosphate buffer (Miwa et al., 1971) was diluted to a final concentration of 0.05 M with respect to $Na^+$. The sample was heated to 90 °C for 10 min, and then cooled and absorbed onto a 1-ml packed bed of BND-cellulose (Serva) equilibrated with 0.01 M Tris-HCl, pH 7.2. The column was washed with 40 ml of the Tris buffer also containing 0.05 M NaCl. Poly(ADP-ribose) free of DNA was recovered by elution with 0.01 M Tris buffer containing 1.0 M NaCl. $MgCl_2$ was added to a final concentration of 10 mM and the resultant precipitate (Doly, 1968) was centrifuged down and resuspended in 1% sodium citrate. Spectral analysis indicated a 230/260 nm ratio of 0.243 and a 280/260 ratio of 0.250. The isolated material had a maximum absorption at 257 and 259 nm at pH 1 and pH 7, respectively, which is consistent with adenine being the chromophore element of the polymer. Thin-layer chromatography (Lehmann et al., 1974) of a venom diesterase digest revealed the presence of the two expected products, 5′AMP and phosphoribosyl-AMP in a ratio of 1:23, indicating a polymer chain length of 24 ADP-ribose units (Nishizuka et al., 1969).

A solution of 0.25 mg of the purified polymer in 1 ml was mixed with 1 mg of methylated bovine serum albumin in complete Freund's adjuvant and injected into the foot pads of the rabbits. Repeat injections of the same antigen in incomplete Freund's adjuvant were made at 1 month intervals. All results presented here were performed with the serum from animals receiving 1 or 2 administered doses of 0.25 mg of poly(ADP-ribose). Control serum was obtained from the same animals before antigen administration. The serum globulin fractions were purified by ammonium sulfate precipitation, Sephadex G100 filtration, and finally by ion exchange chromatography on DEAE-Sephadex (Sober and Peterson, 1958). The fab fragment utilized in some experiments was prepared by papain digestion and ion exchange chromatography (Porter, 1959).

Cell extracts for assessment of poly(ADP-ribose) content were prepared from 25 to 50 × $10^6$ cells as follows. Cells were pelleted from the growth medium and washed once in cold physiological saline. The cell pellet was resuspended in 2 ml of 0.1 N NaOH, incubated for 2 h at 37 °C, and then stored overnight at 4 °C. After neutralization, the sample was adjusted to 10 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.4, 50 mM NaCl, 100 μg/ml of DNase I (Sigma), and 100 units of micrococcal nuclease/ml (Worthington). After 8–10 h of digestion at 37 °C, EDTA was added to a final concentration of 20 mM, and an equal volume of solution containing 150 mM NaCl, 15 mM sodium citrate, 40 mM Tris-HCl, pH 8.0, 0.5% sodium dodecyl sulfate, and 500

Table II: Effect of DNA and Poly(A) on the binding of Poly(ADP-ribose).[a]

| Additions | (cpm) Precipitation |
|---|---|
| None | 253 |
| Poly(A) (1 μg) | 257 |
| Poly(A) (100 μg) | 256 |
| DNA (100 μg, denatured) | 268 |
| DNA (100 μg, native) | 257 |

[a] Assays performed or described in Table I. Poly(A) (Schwarz). DNA, calf thymus, sheared to a chain length of about 3000 bases.

μg/ml of Pronase (nuclease free, Calbiochem) was added. After digestion for 16 h at room temperature, the samples were extracted two times with water-saturated phenol and three times with ether. The residual aqueous solution was used for poly(ADP-ribose) determinations.

Immunoassays for the poly(ADP-ribose) content of cell extracts were performed either by the Farr method (Mider and Farr, 1973) or in a filter disc assay. The latter technique is based on the fact that free poly(ADP-ribose) does not bind to Millipore filters (type HA) while that bound to the antibody or the fab fragment does bind upon filtration. In the Farr assay free polymer remains soluble in 50% saturated ammonium sulfate while the poly(ADP-ribose) bound to the antibody is precipitated.

## Results

The binding of $^{14}$C-poly(ADP-ribose) to the globulin fraction purified from control and immune serum is compared in Figure 1 (filter disc assay method). An antibody in the immune globulin fraction from animals administered poly(ADP-ribose) binds the polymer in a dose-dependent fashion. In contrast, the control globulin fraction displayed little or no affinity for the polymer.

To evaluate the specificity of poly(ADP-ribose) binding to the antibody in the immune serum, various adenine nucleosides and nucleotides were added to tubes containing [$^3$H]poly(ADP-ribose). Aliquots of the immune serum were then added in amounts just sufficient to bind the polymer. After incubation, the immune globulin and any associated poly(ADP-ribose) were precipitated with ammonium sulfate and washed, and the hot PCA extract of the precipitate was counted. As indicated in Table I, none of the compounds tested showed any appreciable inhibition of polymer binding to ammonium sulfate precipitable protein even though these compounds were present at a 25 000-fold excess (w/w) over the amount of poly(ADP-ribose). Similarly, neither DNA nor poly(A) (Schwarz) affected the amount of radioactivity recovered in association with the immune globulin (Table II). Assays in which the immune globulin was omitted indicated that neither poly(A) nor DNA directly binds to poly(ADP-ribose) (filter disc assay). Experiments performed with the globulin fraction from control serum indicated that greater than 95% of the labeled poly(ADP-ribose) was recoverable in the soluble fraction after addition of ammonium sulfate.

The above findings suggested the possibility that the specificity of poly(ADP-ribose) binding to the antibody might be toward the ribose-p-p-ribose moiety of the polymer. Other naturally occurring compounds containing this component were tested for their ability to compete for binding of poly(ADP-ribose) to the antibody. As shown in Figure 2, neither NAD, NADP, NADH, or NADPH appreciably competed even when present in the binding assay at a 100 000-fold excess (w/w) over the amount of labeled poly(ADP-ribose) (Figure 2). In agreement with the results presented in Table I, ADP-ribose did compete with poly(ADP-ribose) for binding. With ADP-ribose present at a 10 000-fold excess, the binding of labeled polymer was reduced by 30% and, at a 100 000-fold excess, the competition was nearly total. Compared with ADP-ribose, unlabeled poly(ADP-ribose) was extremely efficient in reducing the amount of binding of labeled poly(ADP-ribose) (Figure 2). Binding of labeled polymer decreased in a dose-dependent fashion with addition of increasing levels of cold poly(ADP-ribose). One nanogram of unlabeled polymer was sufficient to give a 50% reduction of binding of 1 ng of [$^{14}$C]poly(ADP-ribose).

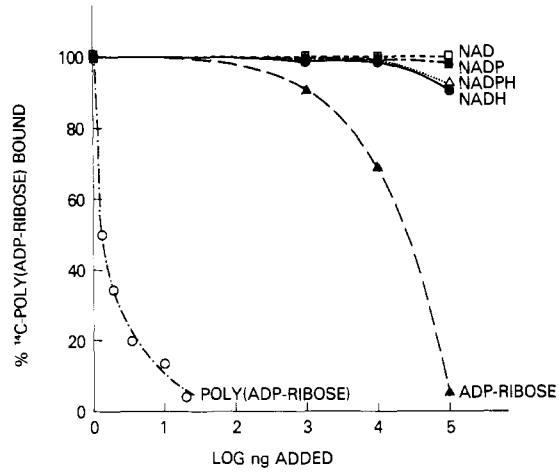


FIGURE 2: Effect of ADP-ribose and ADP-ribose containing compounds on the binding of [$^{14}$C]poly(ADP-ribose). The assay was performed as described in Figure 1 except that 20 μg of an fab preparation from immunized rabbits was used. One nanogram of $^{14}$C-labeled polymer was present in the assay. All nucleotide solutions were freshly prepared.

Based on these observations, competition assays were performed to evaluate the poly(ADP-ribose) content of cells taken at various times after release of the hydroxyurea block. Filter disc assays were performed as described above except that cell extracts (see Methods) were included instead of the nucleosides and nucleotides. Utilizing standard curves constructed from experiments in which unlabeled poly(ADP-ribose) competed with [$^{14}$C]poly(ADP-ribose), the extent of inhibition of binding with cell extracts was directly related to the poly(ADP-ribose) content of the cell extracts. These results are presented in Figure 3. The presumptive poly(ADP-ribose) levels reached a maximum at 4 h after release of the hydroxyurea block and then declined. Then there was a second increase to even greater levels as cells traversed from late S phase to $G_2$ (8 h). By 24 h, the amount of poly(ADP-ribose) returned to basal levels. In a second experiment carried out under identical conditions, the amount of polymer dropped from 3.0 to 1.2 ng/0.2 ml of cell extract as cells progressed from 8 to 10 h after reversal of the hydroxyurea block, indicating the transient nature of the accumulation of poly(ADP-ribose) at the S-$G_2$ transition point. By early $G_1$ (14 h after reversal of the block) there was a further reduction to only 0.8 ng/0.2 ml of cell extract.

To ascertain that the material in cell extracts which competed with labeled poly(ADP-ribose) for binding was really

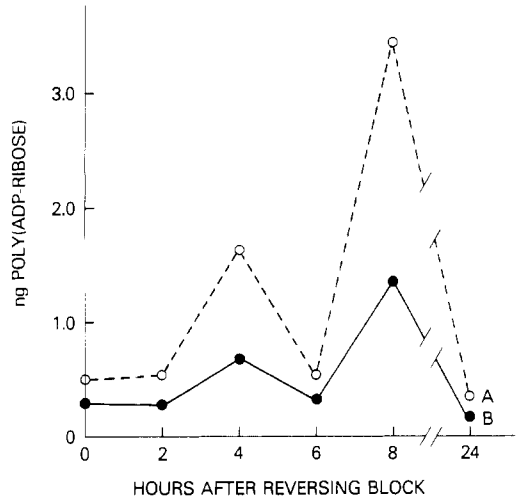


FIGURE 3: Detection of material in cell extracts which inhibits the binding of [$^{14}$C]poly(ADP-ribose) to antibody. Assays were performed as in Figure 2 except that extracts of cells prepared at various times after initiating the S phase were added. Curve A: 0.2 ml of cell extract. Curve B: 0.1 ml of extract. The data are expressed in terms of the mass of unlabeled poly(ADP-ribose) which would produce the same percent inhibition of binding of the labeled polymer as does the cell extract.

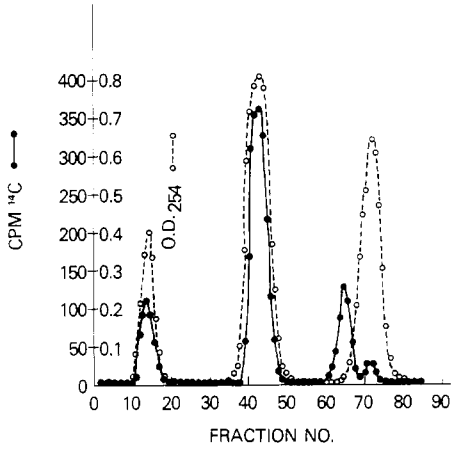


FIGURE 4: Dowex-formate column chromatography of venom diesterase digests. Cells were labeled from 6–8 h after reversal with 0.1 $\mu$Ci/ml of [U-$^{14}$C]ribose (Amersham; 59 mCi/mmol). Cell extracts were prepared in the standard way and incubated for 1 h with 2 mg of immune globulin fraction. The sample was cooled and an equal volume of saturated ammonium sulfate was added. After standing for ½ h at 4 °C, the sample was centrifuged for 30 min at 2000$g$. The recovered pellet was washed five times with cold 50% saturated ammonium sulfate and then resuspended in 2 ml of 0.05 M Tris-acetate buffer, pH 8, containing 0.010 M $MgCl_2$. Following dialysis against the same buffer for 24 h, 1 mg of 5′-AMP and 100/$\mu$g of venom diesterase (Boehringer) were added and the sample was digested for 16 h at 37 °C. After precipitation with cold 5% PCA, the supernatant fraction was recovered, neutralized with KOH, and centrifuged. Aliquots of the supernatant fluid obtained were then adsorbed onto a Dowex-formate column and chromatographed with a formic acid gradient (Ueda et al., 1975).

poly(ADP-ribose), cells were labeled with [$^{14}$C]ribose and cell extracts prepared in the usual way. Radioactive material in the extracts which bound to the antibody was isolated by the Farr method and digested with venom diesterase. Dowex-formate column chromatography (Ueda et al., 1975) indicated the presence of four radioactive peaks (Figure 4). Peak I co-chromatographed with adenosine, peak II with 5′-AMP, and peak IV with ADP-ribose. Peak III which eluted just before an ADP-ribose marker was identified as phosphoribosyl-AMP on the basis of its elution position in this system as well as its mobility on PEI-cellulose plates (Lehmann et al., 1974). Radioactive 5′-AMP is produced by venom diesterase digestion of ADP-ribose and poly(ADP-ribose) while phosphoribosyl-AMP is produced from internal ADP-ribose units of the polymer (Chambon et al., 1966). Adenosine is formed from 5′-AMP by a 5′ nucleotidase which is present in small amounts in the venom diesterases from commercial sources. Thus, all of the radioactivity binding to the antipoly(ADP-ribose) antibody can be accounted for by the poly(ADP-ribose) and ADP-ribose present in the cell extracts.

Since ADP-ribose competes with the polymer for antibody binding, it was necessary to measure the amount of ADP-ribose present in cell extracts to determine what fraction of the competition seen with the extract was due to polymer and what amount due to ADP-ribose. This was done as follows. Cell extracts were prepared from cells at 8 h after the hydroxyurea block, and trace amounts of [$^{14}$C]ADP-ribose were added. Material which co-chromatographed with the radioactive marker on a Dowex-formate column was recovered, lyophilized to dryness, and resuspended in water for spectrophotometric assay. The results indicated that an amount of cell extract which produced a 65% reduction in the binding of labeled polymer (0.2 ml at 8 h, Figure 3) contained only 150 ng of ADP-ribose. From Figure 2 we can conclude that this amount of ADP-ribose would have produced only about 5% inhibition of [$^{14}$C]poly(ADP-ribose) binding. Thus this amount of ADP-ribose would result in an 8% overestimation of the amount of poly(ADP-ribose) present in the cell extract.

Two further observations support the validity of the immunoassay method. First, extracts from cells incubated with [2-$^{3}$H]adenosine contain a labeled material which is converted by venom diesterase into compounds which migrate on Dowex-formate column chromatography as does phosphoribosyl-AMP. Pulse labeling experiments indicate that the amount of radioactivity incorporated into the poly(ADP-ribose) is maximal at 8 h after release of the hydroxyurea block with a smaller peak seen at 4 h (Figure 5). Second, poly(ADP-ribose) polymerase activity as measured in isolated nuclei peaks at 8 h after initiating the S phase.

Although both assay methods indicate that the amount of polymer changes during the cell cycle, the observed differences in amount might be due to differences in recovery of the polymer at the various time points. This is unlikely since exogenously added [$^{14}$C]poly(ADP-ribose) was recovered in the final cell extract in nearly equal amounts (84, 92, and 86% at 6, 8, and 10 h, respectively). The input radioactivity was still present in poly(ADP-ribose) since quantitation by the immunoassay indicated a recovery of 11 460 ng of a total input of 12 890 ng of polymer.

## Discussion

A number of reports have indicated that poly(ADP-ribose) polymerase activity is higher in dividing than in resting cells. Lower extractable enzyme activity is detected in static mouse tissues than in tissue undergoing proliferation (Gill, 1972). Activity is also higher in nuclei from regenerating compared with resting liver (Hilz and Kittler, 1971), in hepatoma nuclei compared with nuclei of normal liver (Burzio and Koide, 1972), and in nuclei from mitogen-stimulated vs. unstimulated lymphocytes (Lehmann et al., 1974). Burzio et al. (1975) also found large differences between the polymerase activity of leukemic cell nuclei and that of nuclei of normal white blood cells in man. [How-

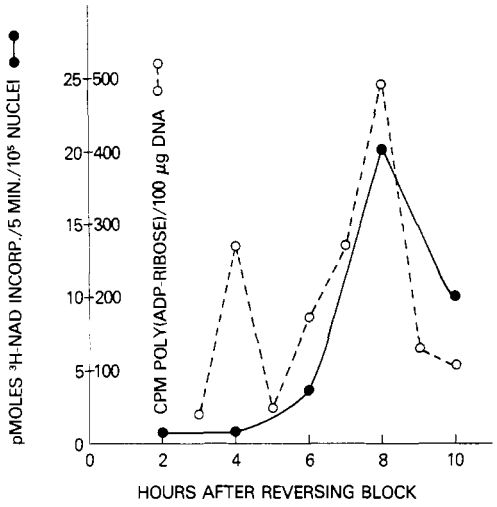


FIGURE 5: Changes in poly(ADP-ribose) polymerase and pulse labeling of poly(ADP-ribose) in synchronous cells. Nuclei were isolated and enzyme activities assessed as described in a previous report (Kidwell and Burdette, 1974). Poly(ADP-ribose) was isolated from cells pulse labeled with [2-$^3$H]adenosine (21 Ci/mmol, 0.6 $\mu$Ci/ml, Schwarz) using a procedure described earlier (Kidwell and Coyler, 1973). In parallel experiments, the poly(ADP-ribose) fraction was shown to be free of RNA contamination ([$^3$H]uridine labeling with 10 Ci/mmol specific activity gave a value of 0.9 cpm/100 $\mu$g of DNA at 8 h). A small DNA contamination was evident since [$^3$H]thymidine labeling (57 Ci/mmol) gave a value of 40 cpm/100 $\mu$g of DNA at 8 h. The radioactive material was not rendered acid soluble by micrococcal nuclease, alkaline phosphatase, DNase I, spleen phosphodiesterase nor RNase A. The fraction was 80% digested by venom diesterase. Dowex-formate column chromatography of the venom diesterase digests indicated that 68–73% of the radioactivity was phosphoribosyl-AMP and 15–20% was 5′-AMP.

ever, for two reports not yet reconciled with the generally observed relationship between growth rate and poly(ADP-ribose) polymerase, see Hilz and Kittler (1971) and Stone and Shall (1975).

When synchronously dividing cells in culture have been employed, poly(ADP-ribose) polymerase activity is found to drop somewhat as cells enter S (Smulson et al., 1971) and then increase as cells progress from S to $G_2$ followed by a drop in activity (Miwa et al., 1973; Kidwell and Watts, 1974; this report). This increase and decrease in enzyme activity in $G_2$ is paralleled by changes in the amount of [$^3$H]adenosine incorporated into the polymer and by changes in the mass of poly(ADP-ribose) as detected by the immunoassay. Equated in terms of the poly(ADP-ribose) standard, there is 200 ng of endogenous polymer per $10^8$ cells at 8 h after reversal of the hydroxyurea block. This is about a six-fold increase over the levels detected in early S phase. These differences, should, however, be considered tentative since it is not known whether the chain length of poly(ADP-ribose) in vivo varies during the cell cycle and how such a variation in chain length would affect the results of the immunoassay. It is probable that short oligomers (dimers, trimers, etc.) might not compete effectively with longer chain length polymers for antibody binding. More information on this aspect of the immunoassay is obviously needed. However, both assay methods give agreement in terms of the pattern of change of poly(ADP-ribose). Also, the fact that chain lengths synthesized in vitro in nuclei with widely differing levels of poly(ADP-ribose) polymerase activity are not significantly different (Burzio et al., 1975) suggests that changes in amount are due to more polymer chains rather than to a difference in chain lengths.

Since the amounts of polymer in cells is so small, conventional methods of quantitation require very large amounts of starting material and this is clearly impractical when dealing with cultured cells. Because the sensitivity of the immunoassay extends into the nanogram range, the assay should be useful for further evaluation of the poly(ADP-ribose) levels in cultured cells in a variety of physiological states.

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