Partial characterization of poly(ADP-ribosyl)ation in the brine shrimp Artemia.

Martin Matthew Gorski
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PARTIAL CHARACTERIZATION OF
POLY(ADP-RIBOSYL)ATION
IN THE BRINE SHRIMP ARTEMIA

by

MARTIN MATTHEW GORSKI

A Thesis
submitted to the
Faculty of Graduate Studies and Research
through the Department of Biological Sciences
in Partial Fulfillment of
the requirements for
the Degree of Master of Science at the
University of Windsor

Windsor, Ontario, Canada

1988
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ISBN 0-315-50511-7
DEDICATION

To my friends and family who were there, who waited, listened, and encouraged me when I could do none of these things, and who shared with me my moments of difficulty and triumph. Thank-you.
ABSTRACT

Poly(ADP-ribosylation)ation is a posttranslational modification encountered in all eukaryotes. It is involved in a variety of molecular processes occurring in the nucleus. The presence of this modification in Artemia was studied and partially characterized.

Artemia embryos at different developmental stages were assayed for activity of the enzyme poly(ADP-ribose) synthetase by measuring the amount of acid-insoluble radioactivity synthesized from [³H]-NAD. The enzyme was present in all embryonic stages and the level of poly(ADP-ribosylation)ation was greatest in embryos just shortly after emergence. The divalent cations Mg²⁺, Mn²⁺, and Ca²⁺ supported the reaction while Zn²⁺ did not. The pH optimum was about 7. A high molecular weight protein (116 kD) and the histone proteins were the main acceptors of poly(ADP-ribose). The pattern and extent of poly(ADP-ribosylation)ation was shown to be dependent on the concentration of [³H]-NAD and the time of reaction. A group of intermediate molecular weight proteins (70 kD - 40 kD) were labeled by β-NAD at low concentrations of the substrate or when incubated with the substrate for not more than 2 minutes. To determine the average chain length and
branching of the newly synthesized polymers, they were stripped from their acceptors by alkali treatment and purified on a dihydroxyboronate column. The polymers were hydrolyzed with snake venom phosphodiesterase and alkaline phosphomonoesterase to yield adenosine, ribosyl-adenosine and diribosyl-adenosine as digestion products. These products were analyzed by reverse phase HPLC. The degree of branching was 40% of the polymer residues for dormant embryos, 10% for 12 hour embryos, 50% for 24 hour embryos and 10% for 48 hour embryos. The average chain length obtained was similar for all developmental stages studied and this was less than one unit long suggesting a high rate of polymer turnover during synthesis.
ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. A. H. Warner, for his guidance and making the pursuit of this thesis possible.

Secondly, I would like to thank the members of my defense committee, Drs. M. J. P. Dufresne, and K. E. Taylor for their time in reviewing this thesis.

In addition, I wish to thank Dr. G. G. Poirier, and Dr. M. K. Jacobson for their generous gifts of time and materials which proved invaluable to the success of this work.

I would also like to thank my colleagues for making the duration of my studies pleasant and exhilarating both in and out of the laboratory.
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<tr>
<td>ADP</td>
<td>adenosine 5'-diphosphate</td>
</tr>
<tr>
<td>5'-AMP</td>
<td>adenosine 5'-monophosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine (5') triphosphate</td>
</tr>
<tr>
<td>dATP</td>
<td>deoxyadenosine 5'-triphosphate</td>
</tr>
<tr>
<td>Ap₄A</td>
<td>diadenosine 5',5'''-P',P₄-tetraphosphate</td>
</tr>
<tr>
<td>Ap₃A</td>
<td>diadenosine 5',5'''-P',P₄-triphosphate</td>
</tr>
<tr>
<td>Ap₂A</td>
<td>diadenosine 5',5'''-P',P₄-pentaphosphate</td>
</tr>
<tr>
<td>BAP</td>
<td>bacterial alkaline phosphatase</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>CPM</td>
<td>counts per minute</td>
</tr>
<tr>
<td>CTP</td>
<td>cytosine 5'-triphosphate</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNase I</td>
<td>deoxyribonuclease I</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>fN</td>
<td>femptonewtons</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine 5'-triphosphate</td>
</tr>
<tr>
<td>Gp₄G</td>
<td>diguanosine 5',5'''-P',P₄-tetratphosphate</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>MNase</td>
<td>micrococcal nuclease</td>
</tr>
<tr>
<td>MNNG</td>
<td>N-methyl-N-nitro-N-nitrosoguanidine</td>
</tr>
<tr>
<td>β-NAD</td>
<td>β-nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NMN</td>
<td>nicotinamide mononucleotide</td>
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PMSF  phenylmethanesulfonyl fluoride
poly(A)  polyadenosine
PR-AMP  phosphoribosyl-adenosine 5'-monophosphate
(Pr)2-AMP  diphosphoribosyl-adenosine 5'-monophosphate
STI  soybean trypsin inhibitor
SV40  Simian virus 40
SVP  snake venom phosphodiesterase
TCA  trichloroacetic acid
TLCK  tosyl-L-lysyl-chloromethyl ketone
UTP  uracil 5'-triphosphate
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INTRODUCTION

Discovery. The molecular events involved in embryonic development and cellular differentiation have not been clearly elucidated. Posttranslational modification of proteins plays an important role in the regulation of enzyme activities and cellular processes. The importance of such modifications in the nucleus of eukaryotic cells with respect to normal growth, development, and differentiation cannot be overemphasized. One such modification is poly(ADP-ribosylation) (for reviews, see Hilz, 1981; Hayashi and Ueda, 1982; Wold and Moldave, 1984; Ueda and Hayashi, 1985).

The synthesis of poly(ADP-ribose) was discovered by two laboratories investigating relatively unrelated projects. Pierre Chambon and his coworkers were studying the synthesis of poly(A) in hen liver nuclei and discovered that the incorporation of radioactivity from labeled ATP into acid insoluble material was stimulated about 1000-fold with the inclusion of NMN, and that the radioactivity was contained in 5'-phosphoribosyl groups, and 5'-AMP (Chambon et al., 1966). Another group working on the biosynthetic pathway of NAD from tryptophan also discovered the
polymer and elucidated its structure (Nishizuka et al., 1967; Hayashi and Ueda, 1982). The enzyme involved was called poly(ADP-ribose) synthetase. It uses β-nicotinamide adenine dinucleotide (β-NAD), which can be thought of as an ADP-ribose moiety covalently attached to nicotinamide, as a substrate to form a homopolymer of ADP-ribose. The molecular structure of this polymer is shown in the diagram on the following page.

**Characteristics.** Poly(ADP-ribose) synthetase was first purified from bovine thymus and was reported to be composed of a single polypeptide having a molecular weight of 130,000 daltons. The $K_m$ and $V_{max}$ for this enzyme with β-NAD as substrate are 60 μM and 0.91 μmol/min/mg protein, respectively, and the required cofactors are Mg$^{2+}$, a thiol group, and DNA (of a minimum size of 220–240 base pairs, or a DNA fraction which copurifies with the enzyme of 10 base pairs) (Yoshihara et al., 1978; Niedergang et al., 1979; Tanaka et al., 1979; Yoshihara et al., 1981). Niedergang and coworkers (1979) purified the synthetase
from calf thymus and showed that both Mn$^{2+}$ and Ca$^{2+}$ could substitute for Mg$^{2+}$ and the optimum pH was 8.

The DNA required for activity was copurified with the enzyme and estimated to be 16 base pairs long and one hundred times more effective than bulk DNA (Niedergang et al., 1979). Another group (when purifying the enzyme) eluted a fraction of DNA from a hydroxyapatite column at 125 mM phosphate that was twenty times more effective in stimulating poly(ADP-ribose) synthetase activity than bulk DNA (Hashida et al., 1979). The efficiency of the DNA used to activate the enzyme correlated with its binding affinity for the enzyme. Heparin and single stranded DNA had strong inhibitory effects on the binding of double stranded DNA to the synthetase. The enzyme was found to have a high frictional ratio (1.81), indicating that it had an elongated shape, or a high solvation, or both. The synthetase was reported to be a basic protein with a pI of 9.8 and amino acid analysis revealed a high lysine content (Ogushi et al., 1980). Although mainly a chromatin associated enzyme, it has been detected in a fraction of rat liver cytoplasm containing endoplasmic reticulum (Adamietz et al., 1981).
Inhibitors. Poly(ADP-ribose) synthetase was inhibited by high ionic strength (Miller, 1986), p-chloromercuribenzoate, ADP-ribose, AMP, and polylysine (Niedergang et al., 1979). Other inhibitors which putatively act at the DNA binding site are pppA2'pA2'pA (Kᵢ=5 μM) (Pizazian et al., 1970), 6-amino-1,2-benzopyrone (6-aminocoumarin) (Hakam et al., 1987), Ap₄A, and ApCH₂pppA (Suzuki et al., 1987). Certain methylxanthines (N⁶-substituted derivatives of adenine) and cytokinins have been found to be competitive inhibitors, these include: nicotinamide, thymidine, theophylline, caffeine, N⁶-(δ²-isopentenyl)-adenine, kinetin-riboside, N⁶-(δ²-isopentenyl)-adenosine, 3-isobutyl-1-methyl-xanthine and quinolinate. Some non-inhibitory analogues are nicotine, and nicotinic acid (Levi et al., 1978). Thymidine was shown by Tanuma et al. (1979) to inhibit the synthetase in vivo and 3-methoxybenzamide and 3-aminobenzamide were discovered as competitive inhibitors with respective Kᵢ's of less than 2 μM (Purnell and Whish, 1979). These latter two inhibitors and nicotinamide were thought to be specific for the synthetase but later were found to inhibit growth, block glucose uptake by cells, induce phosphoenolpyruvate carboxykinase (GTP)
in hepatoma cells, and inhibit nicotinamide methyltransferase in cultured cells at concentrations frequently used in the study of poly(ADP-ribose) synthetase (Grunfeld and Shigenaga, 1984; Leverence et al., 1988; Hunting et al., 1985; Johnson, 1981). The side effects of the above inhibitors may have led to some of the discrepancies and inconsistencies observed in the literature. Aluminum has also been shown to inhibit the synthetase in vitro and in vivo (Crapper et al., 1983). Interestingly, some protease inhibitors at very high concentrations also have inhibitory activity on poly(ADP-ribose) synthetase, namely, TLCK, antipain, leupeptin, and fibrinopeptide A (Cleaver et al., 1986).

**Stimulators.** Single and double stranded breaks in DNA and polyamines have a stimulatory effect on poly(ADP-ribose) synthetase. Spermine and spermidine (1 mM) cause a two-fold increase in poly(ADP-ribosyl)ation of histone H1 in rat liver and alter the poly(ADP-ribosyl)ation pattern of other non-histone proteins (Perella and Lea, 1978; Tanigawa et al., 1980). The stimulation by the polyamines was accompanied by a lower $K_m$ and spermine was more effective than spermidine which, in turn, was more effective than putrescine (Kawamura et al., 1981).
Phenobarbital also has a stimulatory effect on poly(ADP-ribose) synthetase activity (Lechner and Braz, 1985; Braz and Lechner, 1986).

**Peptide Domains and Location of the Synthetase.**

When the enzyme was digested with chymotrypsin, two fragments (66 and 54 kD) were generated. The small fragment (54 kD) was identified to contain the NAD binding domain of the enzyme. The 66 kD fragment, when digested with papain, generated a 22 kD fragment capable of accepting covalently bound poly(ADP-ribose) chains, and a 46 kD fragment capable of binding DNA (Kameshita et al., 1984). Rabbits were injected with synthetase purified from rats and antibodies (IgG) were prepared from the serum which could bind to the enzyme from rat, mouse, and chicken livers and HeLa cells (Ikai and Ueda, 1980). It was observed that "active DNA" could cover one of the antigenic determinants of the protein and a microcomplement fixation method was developed to determine synthetase activity in different tissues (Okazaki et al., 1980a). Monoclonal antibodies were produced against calf thymus poly(ADP-ribose) synthetase which recognized the DNA binding domain of the protein (46 kD fragment). The antibody was found to be cross-reactive with the native enzyme from HeLa cells, mouse testis, and chicken liver and with one of
endogenous degradation products of the enzyme observed in the above tissues (Kameshita et al., 1985). A monoclonal antibody was also produced which recognized the functional domain of the enzyme (Lamarre et al., 1986) and it was used to localize the synthetase on the core nucleosome particles of chromatin by immunogold labeling and electron microscopy (Leduc et al., 1986).

Sources. Poly(ADP-ribose) synthetase has been purified from numerous tissues including hen liver (Tanigawa et al., 1984), and human placenta (Ushiro et al., 1987), and studied in trout testis (Levy-Wilson, 1981), sea urchin embryos (Isoai and Yasumasu, 1985), Ascaris suum (Walter, 1984; Tetrahymena pyriformis (Tsapanakis et al., 1978), Physarum polycephalum (Grobner and Loidyl, 1985; Poirier et al., 1987), Dictyosteilum discoideum (Rickwood and Osman, 1979), Crythecodinium cohnii (Werner et al., 1984), wheat (Whitby et al., 1979), rye (Laroche et al., 1980), and an increasing number of cultured avian and mammalian cell lines. The level of poly(ADP-ribose)lation in crown gall tumor tissue of tobacco was six times greater than that in normal tissue (d'Alessandro and Srivastava, 1985). A recent analysis of poly(ADP-ribose) synthetase activity in a number of higher and lower eukaryotes has also indicated its
presence in some but not all of the organisms tested (Scovassi et al.; 1986). However, the procedure used to solubilize the enzyme may not have been successful due to the structural and biochemical properties of the organism (e.g. shells and cellular inclusions which may bind non-specifically and aggregate the enzyme). Affinity chromatography methods using columns of 3-aminobenzamide (Burtscher et al., 1986) or dihydroxyboronate as ligands has been developed to facilitate the purification and analysis of poly(ADP-ribose) synthetase and poly(ADP-ribosyl)ated proteins (Sims et al., 1980).

**Genetics.** Recently, the gene for poly(ADP-ribose) synthetase from a human hepatoma cell line has been cloned in lambda gt11 (Alkhatib et al., 1987).

**Other Studies.** Most of the work done to date on poly(ADP-ribosylation) has been done on in vitro systems, usually with isolated nuclei or chromatin. It should be noted however that differences between poly(ADP-ribosylation) in vitro and in vivo may be quite pronounced. An 100 to 1000-fold increase in poly(ADP-ribosylation) activity has been reported in broken cells and nuclei compared to intact cells as well as an increase in the number of poly(ADP-ribose) accepting proteins (Tanuma et al., 1985). Studies of
poly(ADP-ribosylation) in vivo are difficult because cells are very impermeable to radiolabeled NAD, and the incorporation of smaller, permeable precursors into other macromolecules complicates the analysis of ADP-ribosylation. Chemical agents have been used to permeabilize cells to NAD, but these agents may introduce artefacts into the cell system. A procedure for delivering radiolabeled NAD into cells using lipid vesicles (liposomes) and avoiding permeabilization associated disturbances has been developed and should prove useful in elucidating poly(ADP-ribosylation) in vivo (Duhl and Hnilica, 1985). Moreover, our understanding of this enzyme system will be enhanced by the use of cell variants deficient in the synthetase (Chatterjee et al., 1987).

Degradation of the Synthetase. Poly(ADP-ribose) synthetase may be degraded in the cell by a number of processes involving proteases. Diadenosine tetraphosphate (Ap₄A) was shown to stimulate the degradation of poly(ADP-ribosylated synthetase into specific fragments in normal human lymphocytes (Surowy and Berger, 1983). Degradation of the synthetase appears to involve an initial cleavage by a cathepsin D-like protease followed by further degradation by a serine protease. However, ADP-ribosylation of the
synthetase had no effect on the course of proteolysis of the enzyme (Surowy and Berger, 1985). Further studies revealed that other nucleotides containing at least a pyrophosphate linkage (such as ATP, dATP, ADP, CTP, GTP, UTP, Ap₃A, Ap₂A, and Ap₁A) were able to stimulate synthetase degradation. An interesting aspect of the nucleotide effect is that both Ap₄A and Gp₄G can be poly(ADP-ribosyl)ated, and that poly(ADP-ribosylation) of these nucleotides is dependent upon the presence of histone H1. Equilibrium dialysis has shown that these nucleotides bind to histone H1 suggesting that a protein-nucleotidé complex may be a functional acceptor unit for a poly(ADP-ribose) chain (Yoshihara and Tanaka, 1981; Tanaka et al., 1981). Furthermore, Zamecnik et al. (1982) have shown that Ap₄A can act as a primer of DNA synthesis by DNA polymerase α, which is active in DNA repair synthesis. Perhaps the regulation of DNA repair synthesis is mediated by the poly(ADP-ribosylation) of the nucleotide-histone H1 complex. This complex can act to stimulate DNA repair synthesis when ADP-ribosylated and poly(ADP-ribose) glycohydrolase can regulate the level of poly(ADP-ribosylation) of the complex. Non-complexed Ap₄A can stimulate the turnover of the synthetase and therefore the level of
poly(ADP-ribosyl)ation in the cell.

Poly(ADP-ribose). The protein acceptors of poly(ADP-ribose) are numerous and include both histones and non-histone proteins. The polymer is covalently attached to protein via an ester bond between the terminal ribose and an internal $\gamma$-COO$^-$ of a glutamyl residue or the $\alpha$-COO$^-$ of the C-terminus (Burzio, 1982; Yan, 1984; Ueda and Hayaishi, 1985). Although all ADP-ribose-protein bonds are labile in dilute alkali, some are labile in neutral $\text{NH}_2\text{OH}$ (Adamietz and Hilz, 1976). It has not been determined which of the three OH groups of the terminal ribose (C1, C2, or C3) could be involved in the protein-ribose bond (Ueda and Hayaishi, 1985). The bonding between consecutive ADP-ribose units involves the 2' OH group of ribose from the AMP moiety (accepting unit) and the 1''-hemiacetal group ribose from the added unit, so it has been assumed that the protein-ribose bond involves the 1''-hemiacetal group. However, recent studies have suggested that bonding may involve the OH groups at the 2'' or 3'' position (Ueda and Hayaishi, 1984; Yan, 1984). Elongation of poly(ADP-ribose) is by the addition of ADP-ribose residues from NAD to the terminus proximal to the synthetase (Ikejima et al., 1987) and the energy required to transfer the
ADP-ribose moiety from NAD to protein is contained in the N-glycoside bond between the nicotinamide and ADP-ribose moieties (−34.3 kJ/mol at pH 7, 25°C; Zatman et al., 1953). The polymer was shown to have a branched structure in vivo involving 2" and 1" OH groups, respectively (Jaurez-Salinas et al., 1982; Kanai et al., 1982). Analysis of the polymer was accomplished by electrophoresis, gel filtration and electron microscopy (Hayaishi et al., 1983), ion exchange chromatography (Kawaichi et al., 1981), and high performance liquid chromatography (Hakam and Kun, 1985). Another technique employed to analyze the average polymer size and number of branch points in vitro involves the digestion of poly(ADP-ribose) with snake venom phosphodiesterase and bacterial alkaline phosphomonoesterase to yield nucleosides (adenosine from polymer termini, ribosyladenosine from internal linear portions, and diribosyladenosine from branch points). The nucleosides may be separated by reverse-phase HPLC, and quantitated by liquid scintillation counting if the polymer was radiolabeled or can be converted to 1-Nε-etheno derivatives prior to chromatography, and detected by fluorescence (Sims et al., 1980). This technique has been used to analyze the low levels of poly(ADP-ribose) in vivo (Jacobson et al.,
1984) and is depicted in the diagram on the following page to illustrate the rationale of the process. Another method of analysis requires the digestion of radiolabeled poly(ADP-ribose) to nucleotides by purified snake venom phosphodiesterase followed by HPLC on a SAX-Partisol column. The digestion products (AMP, PR-AMP, and (PR)2-AMP) can be quantified and used to determine the average chain length and extent of branching (Poirier et al., personal communication).

Another method for analyzing in vivo levels of poly(ADP-ribose) involves radiolabeling of preformed polymers with 3H-borohydride and analysis by reverse-phase HPLC (Hakam et al., 1984, 1986). Antibodies have been made to poly(ADP-ribose) and used for immunofluorescent staining of polymers in cytological sections (Kanai et al., 1981).

Degradation of the Polymer. Poly(ADP-ribose) can be degraded by as many as three different enzymes. Phosphodiesterase from rat liver hydrolyzes the polymer exonucleolytically from the AMP terminus (Matsubara et al., 1970). Poly(ADP-ribose) glycohydrolase is an enzyme specific for poly(ADP-ribose) and was identified in the nucleus and cytosol of HeLa S3 cells (Tanuma et
(From Jacobson et al., 1984)
al., 1986a), and cytosol of human erythrocytes (Tanuma et al., 1986b). The glycohydrolase has been purified from calf thymus and it generates ADP-ribose monomers by cleaving the ribose-ribose bond starting from the chain terminus. In addition, the apparent $K_m$ of the reaction decreases as the polymer length increases (Hatakeyama et al., 1985). Not all poly(ADP-ribosyl)ated proteins are equally susceptible to polymer degradation by the glycohydrolase. Histone H2B is more resistant to polymer hydrolysis in vitro than histones H1 or H2A or protein A24 (histone H2A + ubiquitin covalently joined) (Gaudreau et al., 1986). The protein-ribose bond was hydrolyzed by a another specific enzyme, ADP-ribosyl protein lyase which yields ADP-3' -deoxyxypentose-2'ulose (Okayama et al., 1978; Yan, 1984; Hayaishi et al., 1985). An inheritable (lysosomal storage) disease has been observed where the lesion appears to be in ADP-ribosyl protein lyase. The results of the disease are neurologic degeneration and renal failure (Williams et al., 1984). The half-life of the polymer in permeabilized HeLa cells was found to be 12-15 minutes (Gaal and Pearson, 1986).
The Role of Poly(ADP-Ribosyl)ation in Chromatin Structure. The role of poly(ADP-ribosyl)ation in the eukaryotic nucleus has been shown to be diverse and complex. Initial studies on the distribution of poly(ADP-ribose) synthetase in various functional forms of chromatin have revealed that it is primarily associated with active chromatin (euchromatin) (Mullins et al., 1977; Jump et al., 1979a). It is possible to reconstitute purified synthetase (without DNA) with purified oligonucleosomes and regain enzyme activity (Allan et al., 1979; Jump et al., 1980). Among the acceptor proteins observed for poly(ADP-ribose) are the synthetase itself, and histones H1, H2A, H2B, H3, and A24 (Okayama and Hayashi, 1978; Adamietz et al., 1979; Caplan et al., 1979b; Jump and Smulson, 1980; Kawauchi et al., 1980; Okazaki et al., 1980b; Ogata et al., 1980; Ogata et al., 1981; Adamietz and Rudolph, 1984). It was therefore suggested that poly(ADP-ribosyl)ation plays a role in nucleosome formation and stability. A complex of two histone H1 molecules and an oligomer of 15-16 ADP-ribose units has been observed which supports this notion (Butt et al., 1980; Nolan et al., 1980). Using antibodies to histone H1 and poly(ADP-ribose) the existence of the poly(ADP-ribose)-H1 dimer in vivo has been demonstrated (Wong et
al., 1983). In this case the ADP-ribose polymer is attached to histone H1 at the Cγ of glutamyl residues 2 and 14 (from the N-terminus) and the C-terminal lysine (Ogata et al., 1980; Wong et al., 1984). However, the concentration of NAD has been shown to influence which proteins are ADP-ribosylated and to what extent (Huletsky et al., 1985; Kirsten et al., 1985; Bauer et al., 1986). Indeed, Boulikas (1988) demonstrated more than 60 variant ADP-ribosylated histones in vitro. It has been shown repeatedly that poly(ADP-ribosyl)ation can modulate the superstructure of chromatin. Highly condensed chromatin (30 nm fibre) can be relaxed (10 nm fibre) as a result of poly(ADP-ribosyl)ation of the chromatin proteins and degradation of the polymers restore the condensed 30 nm state (de Murcia et al., 1986). It was also found that in H1-depleted chromatin, histone H2B becomes the main acceptor of poly(ADP-ribose); recondensation of polynucleosomes from H1-depleted chromatin by addition of native H1 was prevented by poly(ADP-ribosyl)ation of the H1-depleted chromatin. Furthermore, antibodies (mono- and polyclonal) specific for histones had greater access to the core histones after poly(ADP-ribosyl)ation of the chromatin, indicating that poly(ADP-ribosyl)ation may be involved in modulating the nucleosomal core
structure of chromatin as well as higher ordered structures (Huletsky et al., 1988). Other studies have shown that the binding of DNA to ADP-ribosylated nucleosomal core particles was relatively weak compared to non-ADP-ribosylated nucleosomes when measured by retention gel analysis. To release the DNA from the particle a force of 60 V (11 fN) was required, whereas that required to displace DNA from control nucleosomal core particles was over 100 V (Mathis and Althaus, 1987). In addition, poly(ADP-ribose) can effectively compete with DNA for binding to histones H3 and H4, suggesting that locally synthesized poly(ADP-ribose) chains may cause partial or complete unwinding of the DNA from the nucleosome core particle (Sauermann and Wesierska-Gadek, 1986; Wesierska-Gadek and Sauermann, 1985).

Modulation of other Enzymes by Poly(ADP-ribosylation). Among the nonhistone proteins capable of accepting poly(ADP-ribose) are DNA polymerase $\alpha$, DNA polymerase $\beta$, terminal deoxynucleotidyl transferase, DNA ligase II, DNA ligase I, ribonuclease, Ca$^{2+}$,Mg$^{2+}$-dependent endonuclease, topoisomerase I and topoisomerase II (Yoshihara et al., 1985; Creissen and Shall, 1982; Ferro, et al., 1984; Darby et al., 1985; Leone et al., 1986; Itaya et al., 1986; Suzuki et al.,
1986; Miller, 1986; Tanaka et al., 1986). Poly(ADP-ribosyl)ation generally resulted in an inhibition of the catalytic activity of these proteins (probably due to an electrostatic repulsion between the protein and DNA) with the exception of DNA ligase II which appeared to be stimulated (Tanaka et al., 1984; Ferro et al., 1984). Other proteins that can be ADP-ribosylated are low mobility and high mobility group proteins from mouse testis nuclei (Mennella et al., 1984), nuclear matrix proteins from cultured mouse fibroblasts (C3H10T1/2) (Wesierska-Gadek and Sauermann, 1985; Cardenas-Corona et al., 1987) and scaffold proteins of HeLa cells (Adolph and Song, 1985a).

**DNA Damage and Repair.** The stimulation of DNA ligation by poly(ADP-ribosyl)ation has been studied extensively. The inhibition of poly(ADP-ribose) synthetase has resulted in a number of conditions in a number of organisms, tissues and cell lines which all seem to involve DNA repair. In general, DNA damage stimulates the activity of the synthetase, although different types of damage elicit different effects on the synthesis of poly(ADP-ribose) (Benjamin and Gill, 1980a; Benjamin and Gill, 1980b; Berger, 1984; Cleaver et al., 1984; Oleinick and Evans, 1984; Wilson et al., 1988). The inhibitor 3-aminobenzamide retarded the
completion of DNA repair, but not DNA repair synthesis, and it was associated with (or immediately prior to) the ligation step of repair (Durkacz et al., 1981; Kreimeyer et al., 1984; Cleaver, 1986; Ireland and Stewart, 1987). Other studies indicated an increased ligation of repair sites upon inhibition of poly(ADP-ribose) synthetase (Miwa et al., 1981; Cleaver and Park, 1986). The discrepancy was attributed to a putative increase in DNA damage caused by the synthetase inhibitor and/or the vehicle used to administer the inhibitor (ethanol) and/or a technical difficulty in accurately measuring low levels of DNA damage (Cleaver and Morgan, 1987). In view of the large amount of literature on this topic, this discrepancy may take considerable time to resolve. Sister chromatid exchanges were increased synergistically in cells by exposing them to methylating and anti-tumor agents in the presence of inhibitors of poly(ADP-ribosyl)ation (Morgan and Cleaver, 1982; Mourelatos et al., 1985; Morris and Heflich, 1984). Active oxygen species may also cause strand disruptions and induce poly(ADP-ribosyl)ation (Hussain et al., 1985; Muelhlematter et al., 1988). If cells are exposed to a lethal dose of hydrogen peroxide, the stimulation of poly(ADP-ribose)
synthetase depletes the NAD and ATP pools leading to cell death. This could be prevented by 3-aminobenzamide (Schraufstatter et al., 1986). Cold shock of CHO cells followed by incubation at 37°C also caused a 3-aminobenzamide-sensitive reduction of cellular NAD (Cantoni et al., 1986) as did heat shock in cultured mouse cells (Jaurez-Salinas et al., 1984).

Poly(ADP-ribose) and Cancer. Poly(ADP-ribosylation) has been implicated in certain processes involved in carcinogenesis and cellular transformation such as in leukemia (Wielckens et al., 1979). When animal cells were exposed to various agents capable of inducing DNA damage (streptozotocin, neocarzinostatin, ionizing radiation, X-rays, methyl nitrosourea, methyl methanesulfonate, or N-methyl-N-nitro-N-nitrosoguanidine), the levels of poly(ADP-ribosylation) were markedly increased (Tamulevicius et al., 1984; Poirier et al., 1985; Stra纳斯, 1984; Jaurez-Salinas et al., 1979; Skidmore et al., 1979; Jump et al., 1979; Nduka et al., 1979). Tumor promoters (eg. PMA and TPA) have also been shown to increase poly(ADP-ribosylation) of nuclear proteins (Singh et al., 1985a; Singh et al., 1985b; Singh et al., 1985c; Singh and Cerutti, 1985; Berger et al., 1987).
DNA damaging agents (alkylating agents, X-radiation and γ-radiation) may not only cause some cancers but they have also been widely used to treat various cancers. Upon exposure to the agent(s), cells attempt to repair the damaged DNA. If the damage is sufficient the high level of poly(ADP-ribosyl)ation depletes the NAD and ATP pools directly leading to cell death. However, if the damage is not sufficient to deplete the NAD/ATP pools, erroneous repair results, leading to malignant transformation of otherwise non-malignant cells. Carcinogen-induced transformation of cells was also suggested to operate in this manner. Therefore, by inhibiting poly(ADP-ribosyl)ation, DNA repair could be halted leading to cessation of cell growth and potentiation of the cytotoxicity of the therapeutic agent(s) (Sugimura and Miwa, 1983; Ben-Hur, 1984; Ben-Hur and Elkind, 1984; Ben-Hur et al., 1984; Borek et al., 1984a; Borek et al., 1984b; Borek et al., 1984c; Shall et al., 1984; Milo et al., 1985; Huet and Laval, 1985; Mourelatos et al., 1986; Thraves et al., 1986a; Thraves et al., 1986b; Berger et al., 1987; Lonn and Lonn, 1987; ). The effects of DNA damage on cells have been shown to interfere with carbohydrate and DNA precursor metabolism (Berger et al., 1986; Das and Berger, 1986; Milam et al., 1986). This may not be
unexpected since NAD, the substrate for poly(ADP-ribosyl)ation, is also important in other metabolic pathways as a coenzyme in cellular redox reactions. Indeed, poly(ADP-ribosyl)ation and NAD may serve as a means to signal changes in metabolic conditions between the nucleus and cytoplasm (Loetscher et al., 1987). It was suggested that ADP-ribosylation may be involved in a cellular suicide pathway which may be triggered by extensive DNA damage. If a cell has sustained sufficient damage it may not be capable of properly repairing itself, and attempting to do so may lead to malignant transformation to the detriment of the entire organism. Since poly(ADP-ribosyl)ation may be stimulated by DNA damage, extensive damage would therefore lead to a rapid depletion of cellular NAD and consequently ATP, ultimately resulting in cell death (cellular altruism or euthenasia) (Wintersberger and Wintersberger, 1985; Alvarez-Gonzalez et al., 1986; Gaal et al., 1987).

Involvement of Poly(ADP-ribosyl)ation in Other Diseases. The involvement of poly(ADP-ribosyl)ation is associated with other diseases such as insulin-dependent diabetes. DNA breaks which are thought to be caused by insulin and epidermal growth factor (Shimizu and Shimizu, 1981) appear to stimulate
poly(ADP-ribose) synthetase therefore depleting cellular NAD and consequently proinsulin synthesis (Yamamoto and Okamoto, 1980; Masiello et al., 1985; Okamoto, 1985). In another case, naturally occurring antibodies to poly(ADP-ribose) have been identified in the serum of patients with systemic lupus erythematosus (Kanai and Sugimura, 1981; Tauchi et al., 1986). The involvement of the antibodies in the disease is unclear, although it was shown that they may also react with single stranded DNA and left-handed Z-DNA (Kanai and Fujiwara, 1984; Kanai et al., 1984; Sibley et al., 1986). Whether the antibodies are produced in response to any of the polynucleotides is also uncertain. Other diseases in which poly(ADP-ribose)lation has been associated are ataxia telangiectasia (Edwards and Taylor, 1980) and xeroderma pigmentosum (McCurry and Jacobson, 1981).

Viruses and Poly(ADP-ribose).
Poly(ADP-ribose)ation has been reported to be involved in the processes of DNA replication and expression controlled by the large T antigen of SV40 transformed cells in vitro and in vivo (Goldman et al., 1981; Baksi et al., 1987). There is also evidence for the involvement of poly(ADP-ribose) in decapsidation and relaxation of adenovirus chromatin. During the late
phase of infection, no histones were found to be ADP-ribosylated, but modified core proteins were assembled into mature viruses. Furthermore, treatment of infected cells with inhibitors of poly(ADP-ribose) synthetase reduced virus particle yield 9-fold, while they reduced infectivity of the particles 10,000-fold (Dery et al., 1985).

**Differentiation.** Poly(ADP-ribosylation)ation may also be involved in reducing the synthesis of random transcripts as shown in vitro (Ohtsuki et al., 1984; Kurl and Jacob, 1985). It has been demonstrated that poly(ADP-ribosylation)ation has antagonistic effects on glucocorticoid induced gene expression. Treating cells with glucocorticoids reduces poly(ADP-ribose) levels and exposure of the treated cells to benzamide and 3-aminobenzamide causes extensive genome degradation and cell death (Kitamura et al., 1979; Kitamura et al., 1980; Wielckens and Delfs, 1986; Johnson and Ralhan, 1986). Treatment of cells with thyroid stimulating hormone was associated with an increase in the activity of poly(ADP-ribose) synthetase (Hepburn and Dumont, 1985). The involvement of poly(ADP-ribosylation)ation in differentiation was studied using Friend erythroleukemia cells and pre-adipocytes in vitro and in rooster spermatogenesis. In all cases, the levels
of poly(ADP-ribose) were decreased during differentiation (Corominas and Mezquita, 1985; Morioka et al., 1980; Morioka et al., 1982; Pekala et al., 1981). Moreover, 3-aminobenzamide enhanced the retinoic acid-induced differentiation of macrophages, granulocytes, promyelocytic leukemia cells in HL-60 cell lines, and EC-A1 cells (murine teratocarcinoma cells) (Damji et al., 1985; Lucas et al., 1984; Ohashi et al., 1984). The inhibitor also stimulated the accumulation of α-lactalbumin in murine mammary tissue implying that reduced poly(ADP-ribosylation) was associated with the differentiation process (Bolander, 1986). Caplan et al. (1979a) indicated a decrease in poly(ADP-ribose) during the early phase of chick limb mesenchymal cell development although the level returned to near normal later during muscle synthesis. However, myogenesis (and chondrogenesis) and formation of syncitia was accompanied by a 3-fold increase in poly(ADP-ribosylation) (perhaps due to strand disruptions in the DNA (Cherney et al., 1985; Farzaneh et al., 1985). It was therefore suggested that the involvement of poly(ADP-ribose) in differentiation is unrelated to DNA synthesis, and that DNA strand breaks may be created in differentiated cells as a mechanism for arresting further mitoses but allowing the early
stages of proliferation (Greer and Kaplan, 1986). It was interesting that the level of poly(ADP-ribosyl)ation remained constant throughout the development of chick lens cells (Counis et al., 1985).

With antibodies to poly(ADP-ribose), immunofluorescent techniques have been used to distinguish normal granulocytes and erythrocytes from lymphocytes and monocytes, and myelocytic leukemia granulocytes and myelocytes from myeloblastic leukemia myelocytes (Ikai et al., 1980). This technique takes advantage of the differences in poly(ADP-ribose) content in cells from different leukemias or stages of differentiation.

Cell Cycle and Poly(ADP-ribose). The activity of poly(ADP-ribose) synthetase was highest at the S and G2 phases of HeLa cells and Physarum polycephalum (Berger et al., 1979; Adolph and Song, 1985c; Grobner and Loidyl, 1985). The number of acceptor proteins was drastically higher in interphase than in metaphase of HeLa cells, and the only acceptor observed at metaphase was the synthetase itself (Adolph and Song, 1985a; Adolph and Song, 1985b; Adolph, 1985; Adolph, 1987a; Adolph, 1987b). In human fibroblasts, susceptibility to transformation by various alkylating agents was highest at S phase and the prevention of transformation in carcinogen-treated cells by exposure to
3-aminobenzamide was maximal also at S phase. These phenomena may have been due to the characteristics of chromatin during S phase during which the structure is relaxed and the DNA has been released from nucleosomes to facilitate synthesis, hence increasing the accessibility of the helix to the chemical (Kun et al., 1983). When C3H10T½ cells were exposed to N-methyl-N-nitro-N-nitrosoguanidine (MNNG), an alkylating agent, and 3-methoxybenzamide, the progression of cells through the cell cycle was prolonged and the cells accumulated in G₂ within the next cycle (Jacobson et al., 1985). In addition, 3-methoxybenzamide strongly enhanced the cytotoxicity of MNNG in exponentially dividing C3H10T½ cells, but had little effect on survival in quiescent cells. Logically, the expression of mutations in growing cells was less than that in quiescent cells since the former tend to accumulate in an irreversible G₂ cell cycle block (Nunbhakdi and Jacobson, 1987).

Artemia. The brine shrimp Artemia is capable of undergoing a remarkable course of events during its embryonic development which makes it a very useful organism to study certain molecular changes during differentiation and growth. In winter egg production, at the onset gastrulation macromolecular synthesis
stops and the embryos become encysted in a tough chitinous shell and dormant (usually in a dessicated state) where they may remain for many decades. Hydration of the dry cysts in sea water leads to a rapid and synchronous resumption of metabolic activity. Protein synthesis begins immediately, and RNA synthesis resumes shortly thereafter. The embryos undergo morphogenesis for about 12 hours and emerge as free nauplius larvae around 16 hours when DNA synthesis resumes (Warner, 1979; Bagshaw, 1982; McClennan and Prescott, 1984; Wahba and Woodley, 1984). The embryos have enough stored food and energy to grow without feeding for up to 72 hours. The purpose of this study was to identify and characterize poly(ADP-ribosyl)ation at different stages of Artemia development and to determine the pattern of protein acceptors of poly(ADP-ribose) prior to and during cellular proliferation.
MATERIALS

Artemia cysts used as a source of poly(ADP-ribose) synthetase in this study were from San Francisco Bay and the Great Salt Lake (Sanders, Ut).

Deoxyribonuclease I (lot Nos. 23C-0181-9 and 93F-0306), micrococcal nuclease (lot No. 19C-0048), m-aminophenylboronic acid hemisulfate, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, morpholinopropanesulfonic acid (MOPS), guanidine hydrochloride (grade I) and β-NAD (lot No. 120C-7400) were from Sigma Chemical Co., St. Louis, Mo; [Adenine-2,8-3H]-NAD (lot Nos. 2300-081 and 2055-202), Enhance (lot No. 036EN7), Biofluor (lot No. 067BF8) and Omnifluor (lot No. 161FB7) were from New England Nuclear, Boston, Ma. Molecular weight markers for electrophoresis were from Biorad, Mississauga, On; hydroxyapatite (Hypatite C) was from the Clarkson Chemical Company Inc., Williamsport, Pa, Bio-gel P 200 (50 - 100 mesh), Bio-gel P 10 (minus 400 mesh) and Bio-Rex 70 (200 - 400 mesh) were from Biorad Laboratories, Richmond, Ca, and Sepharose 6B was from Pharmacia, Uppsala, Sweden. [32P]-NAD (New England Nuclear, Boston, Ma) and a mixture of snake venom phosphodiesterase (SVP), and bacterial alkaline phosphomonoesterase (BAP) (Jacobson et al., 1984) was
a generous gift from G. G. Poirier. It contained
100 units/ml SVP and 10 units/ml BAP in 10 mM MOPS and
50 mM MgCl₂, pH 7.4 (1 unit will hydrolyze 1 µmole of
bis(p-nitrophenyl)phosphate (for SVP) or p-nitrophenyl
phosphate (for BAP) per minute).

Buffers and Solutions. Hatch medium consisted of
422 mM NaCl, 7.2 mM KCl, 22.7 mM MgCl₂·6H₂O, 25.5 mM
MgSO₄·7H₂O, 1.4 mM CaCl₂, 0.5 mM NaHCO₃, 2 g/l borax
(Na₂B₄O₇·10H₂O), 250 I.U./ml penicillin G and 25 µg/ml
streptomycin sulfate. Homogenization buffer contained
250 mM sucrose, 25 mM Tris-HCl, pH 6.5, 25 mM NaCl,
3 mM CaCl₂, and 0.5 mM EDTA. Chromatin preparation
buffers were: Buffer A, 75 mM NaCl, 24 mM EDTA,
pH 8.0; Buffer B, 10 mM Tris HCl, pH 8.0, 0.2 mM EDTA,
0.1 % Triton X-100; Buffer C, 10 mM Tris HCl, pH 8.0,
0.2 mM EDTA; Buffer D, 10 mM Tris HCl, pH 8.0, 0.2 mM
EDTA, and 1.7 M sucrose (57.2 % w/v). The assay
buffer was made up as a 10X concentrate and contained
250 mM Tris HCl, pH 7.4, 250 mM NaCl, 100 mM MgCl₂, and
5 mM EDTA (the concentration of the components in the
assay would be 1/10 of that above). In addition, all
of the buffers contained 0.5 mM DTT, 0.5 mM PMSF and
10 µg/ml STI which were added as 200X stock solutions.
PMSF (0.1 M) was prepared in isopropanol and DTT
(0.1 M) and STI (2 mg/ml) were prepared in water and
stored frozen at -20°C. The mixture of the DNase I and
MNase was prepared as a 50X stock solution in water containing 1 mg/ml DNase I and 10 units/ml MNase. The substrate stock solution was prepared as a 10X concentrate in water and contained 1 mM [³²H]-NAD with a specific activity of 40 cpm/pmole (25 μCi/ml). The [³²P]-NAD substrate stock solution was 65 μM and it had a specific activity of 31 Ci/mmol. Boronate chromatography medium was prepared as follows (according to M. K. Jacobson, personal communication): Twenty-five grams of Bio-Rex 70 was suspended in 100 ml of 0.25 M NaOAc, pH 5.0, and the suspension was adjusted to pH 5.0 by the dropwise addition of concentrated acetic acid. To this 2.5 g of 1-ethyl, 3-(3-dimethylaminopropyl)carbodiimide hydrochloride was added and stirred at room temperature for 15 minutes. 2.5 g of m-aminophenylboronic acid hemisulfate was added to the suspension and the suspension was for another 15 minutes at room temperature. The pH was adjusted to 5.0 as above and stirred for 18 hours at room temperature (periodically monitoring and adjusting the pH, if necessary).
The resin was filtered and washed with the following solutions:

1 l Deionized water
1 l 0.1 M NaOAc, 1.0 M NaCl, pH 4.5
1 l 0.1 M NaHCO₃, 1.0 M NaCl, pH 9.0
500 ml Deionized water
100 ml 0.5 M MOPS, 6 M Guanidine HCl, pH 6.0 (adjusted with NaOH)

The washed resin was resuspended in 50 ml of the 0.5 M MOPS, 6 M guanidine HCl, pH 6.0 and stored at 4°C until required.

Borohate chromatography buffers were: 250 mM ammonium acetate, pH 9.0, 6 M guanidine HCl, and 10 mM EDTA (AAGE-9) and 1 M ammonium bicarbonate, pH 9.0 containing 10 mM EDTA (wash buffer). Reverse-phase HPLC elution buffer was 7% methanol containing 7 mM formic acid.

The scintillation liquids used were 0.4% Omnifluor in toluene for dry heterogeneous samples and Biofluor for aqueous homogeneous samples.
METHODS

Preparation of Artemia Embryos. The cysts were stored at -20°C until used. Dry Artemia cysts were hydrated in 3-5 volumes of 0.5 M NaCl at 0°C for 2 hours. The suspension was diluted with two volumes of cold distilled water and allowed to settle and any floating cysts were removed by suction and discarded. The hydrated cysts were washed with several volumes of cold distilled water and collected on a miracloth filter with a Buchner funnel and washed with several litres of ice-cold distilled water. The washed cysts were weighed and dispensed into 3 litre Fernbach flasks (10 grams cysts per flask). Five hundred millilitres of hatch medium were added to each flask in addition to 1 gram of solid borax. The cysts were incubated with gentle agitation for various periods of time (not exceeding 72 hours) in the presence of an incandescent lamp at 30°C.

The flasks were removed from the incubator at the appropriate times and the Artemia larvae were collected by suction on miracloth with a Buchner funnel. Free swimming nauplius larvae were decanted into a 2 litre separatory funnel leaving any sand or unhatched cysts in the flasks. The separatory funnel was covered with aluminum foil except at the stopcock where a high
intensity lamp was shone to attract the larvae for collection. The larvae were collected in a beaker and filtered through miracloth as above. The larvae were then quick-frozen in liquid nitrogen and stored at -20°C until needed.

**Preparation of Nuclei from Larvae.** All steps were carried out at 0-4°C. Ten grams of frozen larvae were thawed at room temperature until the material was workable. This material was ground by hand in a mortar and pestle with a few strokes to disrupt the larvae shells then suspended in 100 mls of homogenization buffer. This suspension was further homogenized in a loose fitting glass Dounce type homogenizer with ten to twelve strokes of the pestle. The homogenate was filtered by gravity through a cheesecloth-glass wool sandwich filter and centrifuged at 800 x g for ten minutes. The pellet was resuspended in the homogenizing buffer and rehomogenized as above. This material was centrifuged as above, the supernatant fluid was discarded, and the pellet resuspended in the homogenization buffer. A sample of the nuclear preparation was kept for assays and the rest used to prepare chromatin.

**Preparation of Chromatin from Nuclei.** An 800 x g nuclear pellet was homogenized gently by hand in a 50 ml polypropylene centrifuge tube with a teflon
pestle with solution A. The suspension was spun at 1,500 x g for ten minutes. The supernatant fluid was
decanted and saved for other assays. The pellet was
resuspended in solution B with the teflon pestle and
the suspension was centrifuged at 1,500 x g for ten
minutes. The supernatant fluid was discarded and the
pellet resuspended in solution B as above and
centrifuged for ten minutes at 4,500 x g. The
supernatant fluid was discarded and the previous steps
were repeated twice except that centrifugation was
increased to 12,000 x g. The final pellet was
resuspended in 15 ml of solution C and stirred slowly
for 1 hour on ice after which time three equal portions
were layered onto 27 ml of solution D in three 34 ml
ultracentrifuge tubes. The upper two-thirds of the
tubes were mixed and then centrifuged at 70,000 x g for
3 hours in a Beckman SW 25.1 rotor to remove the Triton
X-100 from the preparation. The residual sucrose was
removed from the chromatin pellet by resuspending the
pellet in solution C followed by centrifugation at
30,000 x g for twenty minutes. This process was
repeated and the final sucrose free-pellet was
resuspended in a small volume of solution C and stored
on ice until needed.
Assay of Poly(ADP-Ribose) Synthetase. The synthesis of poly(ADP-ribose) was measured by quantifying the amount of acid-insoluble radioactivity produced upon incubation of the enzyme preparation with \(^{3}H\)-NAD. The number of measurements required in a particular assay determined the total volume of the reaction mixture and therefore the amount of the various reagents required. Since the aliquot size used to measure acid-insoluble radioactivity was 50 µl, the reaction volume was \((50 \text{ µl} \times \text{the number of aliquots}) + 10 \text{ µl}\). A typical reaction mixture requiring 50 µl samples at two time points would contain the following components:

\[
\begin{align*}
84.15 \text{ µl enzyme preparation} \\
11.0 \text{ µl 10X assay buffer} \\
11.0 \text{ µl 1 mM \(^{3}H\)-NAD} \\
2.2 \text{ µl nuclease mixture} \\
0.55 \text{ µl 200X DTT} \\
0.55 \text{ µl 200X STI} \\
0.55 \text{ µl 200X PMSF} \\
110.00 \text{ µl Total Volume}
\end{align*}
\]

The mixture was incubated at 30°C for the desired time period(s) and a 50 µl aliquot(s) was removed and pipetted onto a glass microfibre filter premoistened with 75 µl of 20% trichloroacetic acid. The filters were washed with 5% TCA containing 1 µM unlabeled NAD followed by two washes with 5% TCA. After this step, excess acid was removed from the filters by suction through a sintered glass filter support. The filters were dried in a forced air oven at 60°C. The dried
filters were placed in scintillation vials, covered with 5 mls of Omnifluor scintillation cocktail and counted in a Beckman LS 5801 liquid scintillation counter. To assay chromatin preparations, the procedure was identical to that described above except that chromatin was substituted for nuclei.

**Determination of pH Optimum for Poly(ADP-Ribose) Synthetase from Artemia.** To determine the pH optimum of the enzyme under the described conditions, 10X concentrated reaction buffers (like that described above) were made at different pH's using Tris-HCl as a buffer. The pH's studied were: 5, 6, 7, 8, and 9. Except for the pH, the assay conditions and procedures were identical to those described above. Tris-HCl was used as the buffer in the pH optimum determination.

**Electrophoresis and Fluorography/Autoradiography.** The composition of the gel matrix system used in this study was from Dr. Poirier's laboratory (personal communication). The gel was a composite of polyacrylamide and agarose. The final concentration of the components were 3% acrylamide, 0.5% bisacrylamide, 0.5% agarose, 40 mM phosphate, pH 6.0 (adjusted with solid Tris-HCl) and 6 M urea and 0.1% SDS. The running buffer contained 40 mM phosphate, pH 6.0 and 0.1% SDS. After incubation of the enzyme preparation with \[^{32}P\]-NAD or \[^{3}H\]-NAD, 25% TCA was added to make the
sample 20% with respect to TCA and the preparation was put in an ice bath for ten minutes. Samples were removed and centrifuged at 15,000 x g for 30 minutes. The pellet was redissolved in 100 μl of 90% formic acid, diluted to 1 ml with water and reprecipitated with TCA as above. The sample was centrifuged again for 30 minutes at 15,000 x g. The supernatant fluid was removed and the pellet washed with ether and recentrifuged for ten minutes. The ether washed pellet was dried and dissolved in 40 μl of sample buffer which consisted of 9 M urea, 40 mM phosphate, pH 6.0, 0.2% SDS and 0.01% bromphenol blue as a tracking dye. The sample was applied to a 10 x 10 cm Pab gel and electrophoresed at 4°C and 100 volts until the tracking dye was 2 cm from the bottom of the gel. After electrophoresis, the gel was stained with Coomassie Brilliant Blue, destained with 7.5% methanol and 7.5% acetic acid and photographed. At this time the gel was dried and autoradiographed if the label was \(^{32}\)P or treated with Enhance, dried, and autoradiographed if the label was \(^{3}H\). Gels labeled with \(^{32}\)P required 1-24 hours exposure time and \(^{3}H\) labeled gels were exposed for at least 6 days. The film used was Kodak XAR-5 and the exposures were at -70°C.
Chain Length Determination of Poly(ADP-Ribose)

Synthesized in vitro by Reverse Phase HPLC. To study the size and branching of the free polymer, chromatin was used to make a reaction mixture of 0.25 ml. The specific activity of the $[^3H]$-NAD was increased to about 85 cpm/pmol by the addition of 1 μCi of the stock $[^3H]$-NAD (10 μl) to the reaction tube. This provided a total of 2 μCi of radioactivity in the reaction tube. After the reaction was completed, 1 ml of 25% TCA was added to the tube and the sample was allowed to sit on ice for ten minutes. The precipitated material was centrifuged for 30 minutes at 15,000 x g. The supernatant fluid was discarded and the pellet was redissolved in 100 μl 90% formic acid. This solution was diluted to 1 ml with water and reprecipitated by the addition of 250 μl of 100% (w/v) TCA. The samples were left on ice for ten minutes and centrifuged as above. The pellet was washed twice with ether and allowed to dry. The dry pellet was dissolved in 1 M KOH containing 50 mM EDTA, and incubated at 60°C for 2 hours with occasional mixing. Next, this material was diluted with 10 ml of AAGE-9 buffer (see Materials section) and centrifuged for 20 minutes at 15,000 x g. The supernatant fluid was applied to a BioRex 70-dihydroxyboronate column (0.5-1.0 ml) and the column was washed with 10 ml AAGE-9, followed by 10 ml
of 1 M ammonium bicarbonate, pH 9.0. The polymers were eluted with 5 ml of H₂O and lyophilized. The samples were redissolved in 300 µl H₂O and incubated with the mixture of snake venom phosphodiesterase and bacterial alkaline phosphatase (described in the Materials section) at 37°C for 2 hours in a reaction mixture containing 25 mM ammonium acetate, pH 9.0 and 50 mM MgCl₂. The digested samples were centrifuged to remove particulate material and applied to a reverse-phase column (Altex, ODS) equilibrated with 7% methanol and 7 mM formic acid. The sample was chromatographed isocratically with the above solution at 0.9 ml/min. Fractions of 1 ml were collected in scintillation vials and counted after the addition of 8 ml Biofluor scintillation cocktail.

The average chain length of the newly synthesized polymers was calculated by the ratio of ribosyl-adenosine (which commigrates with deoxyadenosine) to adenosine. The average number of branch points in the molecule was determined by the relative amount of diribosyl-adenosine to total adenosine compounds.
RESULTS

Determination of Poly(ADP-Ribosyl)ation in Nuclei. Samples of nuclei were prepared from hydrated, dormant Artemia cysts, and developing Artemia embryos at 12, 24, and 48 hours of incubation. All nuclei preparations contained yolk platelets due to the physical similarities of the two organelles and relative abundance of yolk platelets to nuclei (Sillero et al., 1980). However, the nuclei were intact as observed by light microscopy using 0.4% methyl green as a stain. The preparations were incubated with 100 μM \[^3\text{H}\text{-NAD as substrate (outlined in "Methods")}}\) and aliquots were removed to determine the incorporation of radioactivity into acid-insoluble material at various times of incubation. The amount of poly(ADP-ribose) synthesized by the nuclei prepared from the various stages of development is shown in Figure 1. The amount of acid-insoluble radioactivity produced by nuclei of developing embryos was greater, in all cases, than that observed for nuclei from dormant embryos (cysts).

Determination of poly(ADP-ribosyl)ation in Chromatin. Chromatin was prepared from the same material used for the nuclei preparation above.
Figure 1. Incorporation of Radioactivity from $[^3\text{H}]$-NAD into Acid-Insoluble Material. Nuclei prepared from hydrated, dormant Artemia cysts (0 hour), and Artemia embryos at 12, 24, and 48 hours incubation were incubated with $[^3\text{H}]$-NAD in a standard ADP-ribosylation reaction. Aliquots (50 µl) were removed at various reaction times and processed as described in Methods.
Figure 2. Distribution of Poly(ADP-Ribose) Synthetase in Soluble and Insoluble Forms. Poly(ADP-ribose) synthetase activity was monitored to determine its release into solution during the preparation of chromatin. Only after the initial homogenization and centrifugation in solution A was there any release of enzyme activity (about 20%) into a soluble state (panel A). After the chromatin was prepared the synthetase remained bound to chromatin and could not be solubilized by extensive digestion of the chromatin by DNase I and MNase (panel B).
During the preparation of chromatin about 20% of the enzyme activity was lost into the supernatant fluid after the initial homogenization of the nuclear pellet in solution A (Figure 2A). After preparation of the chromatin was complete, the enzyme activity remained tightly bound to the chromatin since very little enzyme activity could be released into the supernatant fluid even after extensive digestion of the chromatin with deoxyribonuclease I and micorococcal nuclease (Figure 2B). The amount of synthetase activity associated with the supernatant fluid from the first centrifugation in solution B was not determined due to complete aggregation of the proteins after dialysis in solution C overnight to remove the triton X-100.

To study the potential effect of the nucleases (added routinely during enzyme assays) on the synthesis of acid-insoluble radioactivity from [3H]-NAD, deoxyribonuclease or ribonuclease were added to chromatin reaction mixtures and the amount of acid-insoluble radioactivity synthesized was compared to untreated chromatin. No significant change was observed in the rate and extent of the reaction (Figure 3).
Figure 3. Effect of Nucleases on the Incorporation of Radioactivity from \([^3H]\)-NAD into Acid-Insoluble Material. Chromatin prepared from 24 hour *Artemia* embryos was incubated with \([^3H]\)-NAD in a standard ADP-ribosylation reaction in the presence of DNase or RNase to determine if the nucleases effected the synthesis of acid-insoluble radioactivity. No stimulating effect was observed in the total amount of poly(ADP-ribose) synthesized or the rate of synthesis.
Activity of Poly(ADP-Ribose) Synthetase during Development of Artemia. The incorporation of radioactivity from \(^3\text{H}\)-NAD into acid-insoluble material was compared for chromatin prepared from dormant, 12, 24, and 48 hour Artemia embryos. The concentration of \(^3\text{H}\)-NAD was 100 \(\mu\text{M}\), and aliquots were obtained at 0, 2, 5, 10, and 20 minutes. The results are depicted in Figure 4. The chromatin prepared from 24 hour embryos was the most active and synthesized more poly(ADP-ribose) (acid-insoluble radioactivity) than chromatin from the other stages. Both the initial velocity and total amount of product synthesized were higher for the 24 hour embryos. The total acid-insoluble radioactivity synthesized in 30 minutes by chromatin was compared for various embryo stages and plotted as a function of embryo age (Figure 5). The amount of product synthesized was highest between 24 and 30 hours after the resumption of development.

Requirements for the Poly(ADP-Ribose) Synthetase from Artemia. The effect of \(^3\text{H}\)-NAD concentration on the rate of incorporation of radioactivity into acid-insoluble material was studied with chromatin prepared from 24 hour embryos. The results from two separate experiments are shown in Figure 6. A positive
Figure 4. Incorporation of Radioactivity from $[^3H]$-NAD into Acid-Insoluble Material by Chromatin Prepared from Developing Embryos. Chromatin prepared from hydrated, dormant Artemia cysts (0 hour) and Artemia embryos at 12, 24, and 48 hours of incubation was incubated with $[^3H]$-NAD under standard reaction conditions. The amount of acid-insoluble radioactivity was plotted with respect to reaction time. The chromatin prepared from 24 hour Artemia embryos showed the highest activity.
Figure 5: Poly(ADP-ribosyl)ation in *Artemia* as a Function of Developmental Stage. Poly(ADP-ribose) synthesized by *Artemia* chromatin was measured as the amount of radioactivity from [*H]*-NAD incorporated into acid-insoluble material under standard reaction conditions. *Artemia* embryos at 0, 6, 12, 16, 24, 30, 45, 48, 52, and 72 hours incubation were used as the source of chromatin.
Figure 6. Effect of NAD Concentration on the Activity of Poly(ADP-Ribose) Synthetase. A time course study of the incorporation of radioactivity from [³H]-NAD into acid-insoluble material was done using various concentrations of NAD in an otherwise standard reaction system. Chromatin prepared from 24 hour Artemia embryos was used as the source of the synthetase.

Panel A illustrates the activity of the synthetase in the range of NAD concentrations from 0.1 μM to 100 μM. Panel B illustrates the activity of the synthetase in the range of NAD concentrations from 3 μM to 24 μM. The two experiments (A and B) were done with separate batches of Artemia embryos.
correlation between substrate concentration and enzyme activity was observed. The $K_m$ for $\beta$-NAD as a substrate was found to be in the range of 20 - 60 $\mu$M from analysis of a Lineweaver-Burke plot ($1/(\text{enzyme velocity})$) as a function of $1/(\text{substrate concentration})$.

Since divalent cations have been known to affect the synthetase activity, the effect of four divalent cations was studied using chromatin prepared from 24 hour embryos. The effect of the presence or absence of $\text{Mg}^{2+}$ on the progress of the reaction was studied as well as the effects of $\text{Mn}^{2+}$, $\text{Ca}^{2+}$, or $\text{Zn}^{2+}$. The results (Figure 7) indicated that: 1) divalent cations were required for enzyme activity; 2) not all divalent cations were effective; 3) 10 mM calcium showed the highest activity, followed by 10 mM magnesium, and 10 mM manganese. Manganese was the most effective cation at low concentration and zinc was ineffective at stimulating synthetase activity at either 1 or 10 mM concentrations.

**pH Optimum.** The pH optimum of the poly(ADP-ribose) synthetase reaction was determined by incubating $[^3\text{H}]$-NAD (100 $\mu$M) with chromatin from 24 hour embryos at 30°C in the pH range 5 to 9 and removing aliquots at 0, 10, 30, and 60 minutes
Figure 7. Activity of Poly(ADP-Ribose) Synthetase with Various Divalent Cations. Chromatin prepared from 24 hour Artemia embryos was incubated with [³H]-NAD under standard reaction conditions except Mn²⁺, Ca²⁺, or Zn²⁺ were substituted for Mg²⁺ at concentrations of 1 mM and 10 mM. The reaction volume was 100 µl and incubation was for 30 minutes at 30°C. The total amount of acid-insoluble radioactivity in the reaction mixture was determined.
Figure 8. Determination of pH Optimum. Chromatin was prepared from 48-hour Artemia embryos and used as the source of poly(ADP-ribose) synthetase. Reaction buffers of identical composition but at different pH were prepared and the chromatin samples were incubated in the buffers for one hour at 0°C prior to the enzyme assay. The samples were then incubated with [3H]-NAD under standard reaction conditions and aliquots were removed at 0, 10, 30 and minutes for the determination of acid-insoluble radioactivity. From a plot of CPM vs TIME for each sample, the initial slope was obtained for each pH value and defined as enzyme velocity with the units pmole/aliquot/minute. The velocities obtained were plotted as a function of pH.
incubation to determine the amount of acid-insoluble radioactivity formed. The initial velocities (from the plot of each reaction) were determined and plotted against pH. The results are in Figure 8. These data show that the pH optimum of the synthetase from *Artemia* is about 7.

**Poly(ADP-ribose) Synthetase Storage.** All attempts to store chromatin preparations in the frozen state without loss of synthetase activity were unsuccessful. The inclusion of 10% glycerol or dense sucrose to the stored sample did not prevent the loss of activity upon freezing. In addition, high levels of sucrose inhibited the synthetase activity as shown in Figure 9. Furthermore, if chromatin was digested by nucleases and stored on ice, the enzyme activity diminished to undetectable levels after 2-3 days. However, if the chromatin was left in its native state (i.e., undigested), enzyme activity was still detectable after 10-14 days of storage on ice.

**Effect of Ap₄A and Gp₄G on Poly(ADP-ribosyl)ation.** When diadenosine tetraphosphate (Ap₄A) or diguanosine tetraphosphate (Gp₄G) were included in an otherwise standard reaction mixture at 9.4 mM and 4.5 mM, respectively, the amount of acid-insoluble radioactivity synthesized from
Figure 9. Effect of Sucrose on Activity of Poly(ADP-Ribose) Synthetase. Chromatin prepared from 24 hour *Artemia* embryos was incubated in a standard reaction mixture including 1.7 M sucrose. The time of incubation was 30 minutes and the amount of acid-insoluble radioactivity synthesized from [³H]-NAD was determined.
Figure 10. Effect of Ap₄A and Gp₄G on the Incorporation of Radioactivity into Acid-Insoluble Material. Chromatin was prepared from 24 hour *Artemia* embryos and used as a source of synthetase. Concentrated Ap₄A and Gp₄G were added to a standard reaction mixture. The final concentrations of the dinucleotides is given in the figure legend. Incorporation of radioactivity from [³H]-NAD into acid-insoluble material was measured at times 0, 2, 5, 10, and 20 minutes incubation. The radioactivity is expressed as cpm/50 µl aliquot of the reaction mixture.
[\textsuperscript{3}H]-NAD was reduced. These results are shown in Figure 10. It was not determined if the dinucleotides were acceptors of ADP-ribose. This would have involved separation by ion exchange HPLC and liquid scintillation counting. It is very difficult however, to remove unreacted [\textsuperscript{3}H]-NAD from the reaction mixture without removing small tri- and perhaps tetranucleotides which are the subject of analysis.
Attempts to Purify the Poly(ADP-Ribose) Synthetase

Hydroxyapatite Chromatography. Several attempts were made to achieve purification of poly(ADP-ribose) synthetase from Artemia, by chromatography on a column of hydroxyapatite using a potassium phosphate gradient in 1 M KCl. High salt (on the order of 1 M) was required to solubilize the enzyme for chromatography. Nuclei were prepared from 10 grams of 24 hour embryos and incubated at 30°C with a mixture of DNase I and MNase with 1 mM MnCl₂ for 15 minutes (Wiberg, 1958) and for an additional 15 minutes at 30°C with 10 mM MgCl₂. This preparation was then stirred with 50 mM potassium phosphate, pH 7.4, containing 1 M KCl for 2 hours on ice. The nuclease digestion step was carried out so that the viscosity of the solubilized enzyme preparation was low enough to facilitate chromatography. The preparation was centrifuged at 12,000 x g for 20 minutes to removed particulate material and the supernatant fluid was kept for chromatography on hydroxyapatite. The hydroxyapatite was "defined" several times in distilled water before it was packed by gravity into a 1.5 x 10 cm column, and equilibrated with a solution 1 M KCl buffered with 50 mM potassium phosphate, pH 7.4. A peristaltic pump was used to maintain a flowrate of 0.2 ml/min. The sample was pumped onto the column at the same flowrate and
unbound material was washed from the column with 25 ml of the equilibration buffer. The bound material was eluted from the column with a 100 ml linear gradient of potassium phosphate (pH 7.4) from 50 mM to 500 mM. The concentration of KCl was kept at 1 M throughout the procedure. Fractions of 4.5 mls were collected and analyzed at 260 and 280 nm in a spectrophotometer (Beckman Model 25). The fractions were pooled for concentration using an Amicon PM 10 ultrafiltration membrane, then dialyzed against solution C overnight prior to assay for poly(ADP-ribose) synthetase activity. The optical density of the fractions was plotted as a function of eluant volume and the synthetase activity from the pooled fractions was superimposed on the elution profile. These data are shown in Figure 11. The enzyme activity eluted from the column in a broad peak from 85 mM to 125 mM potassium phosphate.

**Biogel P 200 Gel Filtration.** Separation of the synthetase from nucleic acids was attempted by gel filtration using a Biogel P-200 column. The concentrated active fraction obtained from
Figure 11. Fractionation of Poly(ADP-Ribose) Synthetase Activity on a Hydroxyapatite Column. The procedure is described in the text. The fractions were pooled as indicated, concentrated, and dialyzed against solution C at 4°C prior to the assay. The enzyme activity was expressed as CPM of acid-insoluble radioactivity in 50 µl of the reaction mixture. The concentration of potassium phosphate is given on the second right axis as mM and indicated by the straight line.
hydroxyapatite chromatography, after dialysis in 25 mM potassium phosphate (pH 7.4) and 1 M KCl, was applied to the Biogel P 200 column (2.5 cm x 33 cm) equilibrated with the same buffer. The column was eluted at 10 ml/hour and 5 ml fractions were collected for spectrophotometric analysis. Fractions were pooled, concentrated and dialyzed prior to assay for synthetase activity which was found to remain in the high molecular weight material excluded from the gel matrix beads. See Figure 12.

**Sepharose 6B Gel Filtration.** Gel filtration of the *Artemia* poly(ADP-ribose) synthetase prepared from nuclease digested chromatin, before chromatography on hydroxyapatite, was attempted on Sepharose 6B which has a higher exclusion limit than Biogel P 200. Native (undigested) chromatin which could not readily be sieved due to a prohibitively high viscosity was suspended in solution C and digested with a mixture of DNase I and MNase with 1 mM MnCl₂ for 15 minutes at 30°C (Wiberg, 1958) and for an additional 15 minutes at 30°C with 10 mM MgCl₂. The digested material was made 1 M with respect to NaCl, mixed by vortexing and then centrifuged at 12,000 x g for ten minutes. The sample was chromatographed on a column of Sepharose 6B with the same buffer (solution C plus 1 M NaCl),
Figure 12. Chromatography of a Crude Poly(ADP-Ribose) Synthetase Preparation on a Column of Biogel P 200.
The procedure is outlined in the text. Fractions were pooled as indicated, concentrated, and dialyzed against solution C at 4°C prior to the assay. The enzyme activity is shown as CPM/50 µl aliquot of the reaction mixture.
fractions were analyzed in a spectrophotometer at 260 and 280 nm and plotted as a function of eluant volume.

Selected fractions were pooled, concentrated, dialyzed overnight in solution C and assayed for synthetase activity. The results are shown in Figure 13. The top panel illustrates the elution profile of native chromatin. Most of the U.V.-absorbing material comes off the column in the molecular weight range of 1-2 million daltons. After digestion with the mixture of nucleases, the material elutes from the column at or near the low molecular weight cut-off point of the column (lower panel). The poly(ADP-ribose) activity, eluted at a molecular weight range of 100,000 - 200,000 daltons. Further gel filtration was not attempted since the enzyme was still insoluble in low ionic strength buffers and the synthetase activity was very unstable after digestion and chromatography.

Demonstration of Acceptor Proteins for Poly(ADP-Ribosyl)ation. Due to the overwhelming predominance of yolk proteins in preparations of *Artemia* nuclei, nuclear preparations were not suitable for electrophoretic studies to demonstrate the presence and properties of acceptor proteins for poly(ADP-ribose). Therefore, chromatin was used as a source of enzyme and
Figure 13. Chromatography of Poly(ADP-ribose) Synthetase from *Artemia* Chromatin on a Column of Sepharose 6B. The procedure is outlined in the text. Panel A shows the U.V. absorption profile for *Artemia* chromatin chromatographed before treatment by DNase I and MNase. Panel B shows the U.V. absorption profile for *Artemia* chromatin chromatographed treatment by DNase I and MNase. Fractions were pooled as indicated, concentrated, and dialyzed against solution C at 4°C prior to the assay. The amount of acid-insoluble radioactivity synthesized was determined for each pooled fraction and expressed as CPM/50 µl reaction mixture.
LEGEND

- A-280
- A-260
- CPM

Absorbance

VOLUME (ml)

CPM
potential acceptors. Chromatin prepared from Artemia of various developmental stages was incubated with either $[^32P]-NAD$ or $[^3H]-NAD$. Total acid-insoluble material was solubilized and electrophoresed as outlined in the Methods. The electrophoresis gels, after photography, were either autoradiographed or fluorographed. The pattern of poly(ADP-ribosylation) was found to be different at various concentrations of NAD (Figure 14). At 0.1 μM NAD, histones and a broad band slightly above the synthetase were radiolabeled. In addition, the synthetase appeared to be labeled and formed a discrete band along with another band above the histone group which may be an H1-dimer (lane 1). At 1.0 μM NAD three additional radiolabeled bands of intermediate molecular weight appeared (lane 2), but disappeared at NAD concentrations of 10-100 μM (lanes 3-4). Furthermore, in a time course study of protein modification by poly(ADP-ribosylation) with $[^32P]-NAD$ at 100 μM, a change in the radiolabeling pattern of the acceptor proteins was observed (Figure 14). The ADP-ribosylation reaction was stopped at times 0, 2, 10, and 30 minutes. Radiolabeling at time zero was near background (lane 5, 14B), and at 2 minutes at least eight species of protein were radiolabeled (lane 6, 14B). However, at 10 minutes incubation a new
Figure 14. Electrophoresis and Autoradiography of ADP-Ribosylated Chromatin Proteins. Chromatin prepared from 24 hour Artemia embryos was incubated with $^{32}$P-NAD as described in "Methods". Panel A shows a photograph of the coomassie stained gel. Panel B shows the autoradiogram of a 6 hour exposure. Lanes 1-4 show the effects of NAD concentration on the pattern of poly(ADP-ribose)lation. The concentrations of NAD used were: 0.1 μM, lane 1; 1 μM, lane 2; 10 μM, lane 3; and 100 μM, lane 4. Lanes 5 - 8 show the time course of incorporation of radioactivity from $^{32}$P-NAD into proteins from the prepared chromatin. The reaction volume was 220 μl and 50 μl aliquots were used as samples for electrophoresis. The time points sampled were: 0 minutes, lane 5; 2 minutes, lane 6; 10 minutes, lane 7; and 30 minutes lane 8. Lane MW (panel A) shows the migration of the molecular weight markers which were: β-galactosidase (116 kD), phosphorylase B (97 kD), bovine serum albumin (66 kD), ovalbumin (42 kD), and soybean trypsin inhibitor (27 kD).
radiolabeled band appeared and four labeled bands from the 2 minute aliquot disappeared (lane 7, 14B). By 30 minutes, the only radiolabeled bands observed were those for the histone proteins, the synthetase, and a band of greater molecular weight than the synthetase (lane 8, 14B). The time course study using \(^{3}H\)-NAD was not as revealing (Figure 15B, lanes 5-9) as that with \(^{3}P\)-NAD perhaps because of the lower energy of tritium decay. When chromatin prepared from Artemia at different developmental stages was ADP-ribosylated with \(^{3}H\)-NAD, a drastic change in the acceptor protein distribution was observed (Figure 15, lanes 1 - 4). In the earlier stages of development (prior to emergence and DNA synthesis), a number of proteins were acceptors of poly(ADP-ribose); including most of those seen with 1 µM NAD and at 2 minutes with 100 µM NAD (lanes 1 and 2). In the latter stages of development (after emergence and initiation of DNA replication) only the histone group and the synthetase were radiolabeled to any extent (lanes 3 and 4). These observations will be discussed below.

Physical Structure of Poly(ADP-Ribose) Synthesized by Artemia. The average polymer chain length of poly(ADP-ribose) and degree of branching was determined
Figure 15. Electrophoresis and Fluorography of ADP-Ribosylated Chromatin Proteins. Chromatin was prepared from Artemia embryos incubated for 0, 12, 24, and 48 hours to study changes in the pattern of poly(ADP-ribosylation) of chromatin associated proteins as described in Methods. In panel A (coomassie stained gel) the presence and distribution of the various protein species from chromatin for Artemia embryos at different stages appears to be the same. The embryo ages used in this study were: 0 hour, lane 1; 12 hour, lane 2; 24 hour, lanes 3 and 5 - 10; and 48 hour, lane 4. Panel B shows the incorporation of radioactivity from [\(^{3}\text{H}\)]-NAD into these proteins under standard reaction conditions. Lanes 5 - 10 show a time course of incorporation of radioactivity from [\(^{3}\text{H}\)]-NAD into chromatin associated proteins from 24 hour Artemia embryos. The time points sampled were: 0 minutes (lane 5), 2 minutes (lane 6), 5 minutes (lane 7), 10 minutes (lane 8), 20 minutes (lane 9), and 45 minutes (lane 10). The gel was treated with Enhance, dried, and exposed to Kodak X-AR film for 7 days. Lanes 2 and 5 contained less of the reacted material than the other lanes.
for reactions catalyzed by the synthetase prepared from embryos at various stages of Artemia development. The analytical technique required purifying poly(ADP-ribose) polymers, digesting them to nucleosides and separating the digestion products by reverse-phase HPLC. The results are depicted in Figure 16. The average chain length for all chromatin preparations tested appeared to be less than one unit determined by the ratio of ribosyl-adenosine to adenosine. Indeed, the data indicate a length between 0.1 and 0.3 ADP-ribose units as shown in Figure 17. This low value was probably due, in part, to some residual [³H]-NAD in the second TCA precipitation step in the Methods section. The contaminating [³H]-NAD would be hydrolyzed to adenosine and ribosyl-nicotinamide causing an overestimation of adenosine allegedly obtained from poly(ADP-ribose) digestion. Degradation of the polymer during synthesis incubation by a glycohydrolase (perhaps poly(ADP-ribose) glycohydrolase) may also have contributed to the apparently shortened chains. This possibility is discussed in greater detail later. The short chain lengths were not due to hydrolysis caused by contamination of the nucleases routinely used during
Figure 16. Nucleoside Composition of Digested Poly(ADP-Ribose). Chromatin was prepared from Artemia embryos at various developmental stages and incubated with [3H]-NAD under standard reaction conditions in a total reaction volume of 300 µl. The poly(ADP-ribose) synthesized was purified, digested, and chromatographed on a reverse-phase HPLC column as described in "Methods". The radioactivity in the fractions was determined by liquid scintillation counting using 8 mls of Biofluor for each 1 ml column fraction. The radioactivity of the fractions (CPM) was summed for the A254 peaks representing the nucleoside digestion products for the samples prepared from the various stages of development.
Figure 17. Chain Length and Branching of Poly(ADB-Ribose). The radioactive counts obtained and shown in Figure 16 were expressed as quantities relative to each other (i.e., the ratio of ribosyl-adenosine : adenosine or diribosyl-adenosine : total adenosine. The ratio of ribosyl-adenosine to adenosine was used as an indication of average chain length and the ratio of diribosyl-adenosine to the sum of ribosyl-adenosine and adenosine was used to indicate the average degree of branching.
Figure 18. Purity of Nucleases used in the Synthesis Reaction of Poly(ADP-Ribose). The presence of contaminating phosphatases in the nuclease mixture routinely used in the standard reaction mixture was checked by incubating ATP with the nuclease preparation at 30°C for 15 minutes. The sample was chromatographed on a reverse-phase HPLC column (Altex, ODS) and the eluted products compared to samples of AMP, ADP, ATP chromatographed on the same column. Panel A shows the elution profile of AMP, panel B shows the elution profile of ATP, panel C shows the elution profile of ADP and panel D shows the elution profile of ATP after incubation with the nuclease mixture.
the standard reaction incubation of chromatin with [\(^3\)H]-NAD since the nucleases had no effect on ATP (which also contains phosphodiester bonds) (Figure 18).
DISCUSSION

The enzyme poly(ADP-ribose) synthetase was found to be tightly associated with chromatin in Artemia embryos and behaved comparably with poly(ADP-ribose) synthetase prepared from other sources as outlined in the Introduction. The pH optimum around 7 was slightly lower than that published for the synthetase from other sources, but the requirement for divalent cations was the same as that described elsewhere (Niedergang et al., 1979). However, the biochemical and biophysical properties of poly(ADP-ribose) synthetase from Artemia would best be elucidated using purified enzyme. The results obtained from studying nuclei prepared from Artemia embryos were dubious. This was primarily because of the predominance of yolk platelets in the preparations of nuclei. Even chromatin prepared from the nuclei preparations was not completely free of yolk proteins indicated by the dark brown colour of the chromatin. Recently however, a technique has been developed to purify nuclei from Artemia, free from yolk platelets (Squires et al., 1988). This technique would prove very useful in studying poly(ADP-ribosyl)ation in Artemia nuclei in vitro since interference caused by yolk platelets would be minimized.
Purification of poly(ADP-ribose) synthetase from Artemia was unsuccessful, although a fraction of the synthetase activity was enriched after nuclease digestion and gel filtration on Sepharose 6B. Further attempts to purify the enzyme should involve DNA affinity chromatography and dihydroxyboronate chromatography.

The data obtained by studying poly(ADP-ribose) synthetase from Artemia chromatin in vitro should be approached with caution because there may be protein acceptors of poly(ADP-ribose) in the nuclei of Artemia which are not present in the chromatin preparations. The chromatin preparations used are devoid of many proteins normally found in the nucleus because they do not bind to DNA and are lost during the chromatin isolation procedure. Therefore, the pattern of acceptor proteins in situ may be different from that demonstrated here with chromatin preparations in vitro.

The patterns of poly(ADP-ribosyl)ation in chromatin from Artemia prepared at different developmental stages were quite interesting. The differences observed in chromatin were not observed using whole nuclei prepared from the same stages or from the same samples of Artemia embryos. This observation was most likely due to reduced assay
efficiency in the nuclei preparations by effects of the yolk platelets and yolk proteins on the synthesis of poly(ADP-ribose) and colour quenching of the reaction aliquots caused by the accompanying pigment during liquid scintillation counting. Using chromatin preparations, the amount of poly(ADP-ribose) (acid-insoluble radioactivity) synthesized and the rate of synthesis were higher in chromatin from 24 hour embryos than in either pre-emerged or older (48 hour) embryos. Emergence of Artemia embryos occurs at about 16 hours after which time DNA replication is re-initiated and mitosis resumed. The observation of higher poly(ADP-ribose) synthesis in 24 hour embryos (undergoing DNA synthesis, i.e. in the S phase of the cell cycle) is in agreement with that made by researchers studying HeLa cells and Physarum polycephalum (Berger et al., 1979; Adolph and Song, 1985c; Grobner and Loidyl, 1985).

The chain lengths observed in the various Artemia preparations were all much less than one unit long, but the polymers showed a considerable amount of branching, as indicated by the relative abundance of diribosyl-adenosine in the digestion products of hydrolyzed poly(ADP-ribose). It has been reported that poly(ADP-ribose) has a branched structure in vivo and that branching only occurs in polymers of substantial
length (Kanai et al., 1982; Juarez-Salinas et al., 1982). Therefore, hydrolysis of the polymer during the synthesis reaction was suspected. In order to generate the proportions of nucleosides observed from poly(ADP-ribose), the ribose-ribose bond of the internal linear residues must be cleaved. This reaction has been shown to be catalyzed by poly(ADP-ribose) glycohydrolase (Hayaishi and Ueda, 1982) and its involvement in these studies cannot be ruled out. Other glycohydrolases may also be involved, but it is unlikely that non-enzymatic degradation of this glycoside bond occurs because of its relatively high stability. The apparent amount of branching of poly(ADP-ribose) prepared from Artemia seemed rather high. Indeed, other studies have indicated amounts of polymer branching on the order of 2%, or one branch point in 50 linear ADP-ribose residues (Kanai et al., 1982; Juarez-Salinas et al., 1982). The significance of this apparent divergence from the norm is unclear. Given the structural and biochemical similarities of poly(ADP-ribose) synthetase prepared from various sources, it is difficult to accept the unusually high amount of branching as a result of synthesis by the synthetase from Artemia. Rather, it is tempting to speculate about a correlation between the abnormally low (apparent) internal linear residues and the
abnormally abundant branching residues to explain the observations but an explanation is not forthcoming.

The distribution of poly(ADP-ribose) acceptor proteins in embryos from different developmental stages was similar to that described elsewhere (Adolph and Song, 1985a; Adolph and Song, 1985b; Adolph, 1985; Adolph, 1987a; Adolph, 1987b). In pre-emerged embryos, acceptor proteins for poly(ADP-ribosyl)ation were numerous. Upon emergence (and entry into S phase), the predominant and perhaps only acceptors were the synthetase itself and the group of histone proteins. This does not necessarily mean that other proteins were not poly(ADP-ribosyl)ated in the reaction mixture. It may mean that the stability of any poly(ADP-ribose) attached to them is considerably less than that attached to the synthetase and histones. Perhaps the turnover rate of protein-bound poly(ADP-ribose) is specific for the various acceptors or classes of acceptors, depending upon their conformational and molecular similarities or dissimilarities. The possibility of high poly(ADP-ribose) turnover was indirectly indicated by the data of the chain length and degree of branching of the polymer. To further the evidence for polymer turnover, it was shown that different proteins became modified and subsequently unmodified during a time course investigation of
poly(ADP-ribose) synthesis (see Figure 15). In addition, the concentration of NAD used in the synthesis reaction resulted in differential modification of acceptor proteins. At concentrations of 1 μM, a greater number of acceptor proteins was observed than at 10 and 100 μM NAD. It is interesting to note that the pattern of poly(ADP-ribosylation) at 0.1 μM NAD resembled that obtained at the higher NAD concentrations. Therefore the change in poly(ADP-ribosylation) patterns at different NAD concentrations probably represents differences in the turnover rate of the polymer attached to various proteins rather than differences in the \( K_m \) of the acceptor proteins.

Another possible explanation for the difference in poly(ADP-ribosylation) patterns for chromatin prepared from *Aristea* at different stages of development is that the acceptor proteins detected in the 0 and 12 hour embryos are not present in chromatin prepared from 24 or 48 hour embryos. Indeed, the acceptor proteins seen in the chromatin prepared from the early stages of development are probably DNA binding proteins. The function of these proteins in the regulation of DNA metabolism may be attributed to their binding to DNA. It has been suggested that ADP-ribosylation may coordinate DNA excision repair, DNA replication and
cell cycle progression (Malaise, 1984). Even in the absence of such a putative function for these proteins, the replication of DNA requires the relaxation of chromatin structure and dissociation of DNA from its binding proteins. The absence of the poly(ADP-ribose) acceptor proteins after embryo emergence then, may simply be due to the normal dissociation of these proteins from DNA upon initiation of DNA replication.

The involvement of Ap4A in poly(ADP-ribose)ylation was obscure in these studies. Purified poly(ADP-ribose) synthetase and careful reconstitution studies may be of some assistance in clarifying any relationship between the two.

Future studies of poly(ADP-ribose)ylation in *Artemia* should most likely include identification of the acceptor proteins and direct demonstration of poly(ADP-ribose) turnover as a function of developmental stage. Purification of poly(ADP-ribose) synthetase and poly(ADP-ribose) glycohydrolase from *Artemia* would be required for reconstitution studies. Antibodies directed against purified DNA metabolizing enzymes (particularly those reported to be acceptors of poly(ADP-ribose)) may be used to screen the acceptor proteins for identification. The preparation of antibodies for this purpose however, must be such that none are present which react with the antigens' DNA
binding domain since these domains probably share biochemical and conformational properties leading to potential crossreactivity.

These studies have shown that the brine shrimp _Artemia_ has poly(ADP-ribose) synthetase activity located in the nucleus. They also indicate a role for poly(ADP-ribosyl)ation in the normal progression of _Artemia_ embryos through differentiation and development. The data observed, with the exception of poly(ADP-ribose) chain length and branching, are similar to those reported for poly(ADP-ribose) synthetase prepared from various other sources.
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