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POLY(ADP-RIBOSE) POLYMERASE:
ASPECTS OF ITS INTERACTION WITH DNA

by

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Submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

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ABSTRACT

Poly(ADP-ribose) polymerase from calf thymus was purified to near homogeneity. The use of red-agarose resulted in a rapid purification with a high yield. Purified poly(ADP-ribose) polymerase was inhibited by 1,10-phenanthroline, a metal chelating agent, at $\text{pH} < 8$. This inhibition and the inhibition by other chelating agents suggested that this enzyme was a metalloprotein. Control experiments eliminated the possibility that the inhibition was due to the DNA-degrading properties of 1,10-phenanthroline. Atomic absorption spectroscopy showed the presence of one atom of zinc per protein molecule. Dialysis of the enzyme against buffers containing 1,10-phenanthroline resulted in the loss of activity and the coincidental removal of zinc from the enzyme. Initial rate kinetics showed that 1,10-phenanthroline was non-competitive with NAD^+ and competitive with DNA. The binding of DNA to the enzyme was unaffected by the inhibitor. These results suggest a metal-containing site is involved in the interaction of DNA (and poly(ADP-ribose) polymerase.

Previous reports have indicated that poly(ADP-ribose) polymerase, besides modifying various chromatin proteins, also modifies itself. The studies reported here indicate that this auto-modification inhibited the enzyme. By observing the binding of poly(ADP-ribose) polymerase to the DNA it was shown that the affinity of modified poly(ADP-

ribose) polymerase for DNA was decreased. Mg^{2+} and histone H1 appear to activate the polymerase by increasing the affinity of the auto-modified polymerase for DNA, probably by neutralizing the negative charges on poly(ADP-ribose). The coupling of poly(ADP-ribose) glycohydrolase with the polymerase reactivated the polymerase by degrading the poly(ADP-ribose) and restoring the polymerase-DNA complex. These results provided the basis for a shuttle mechanism by which proteins could be moved on and off DNA by the actions of poly(ADP-ribose), polymerase and glycohydrolase.

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INTRODUCTION

Poly(ADP-ribosyl)ation is an unusual form of covalent post-translational modification. In this reaction the degradation of NAD^+ is followed by a transfer of the ADP-ribose moiety to a protein acceptor and release of nicotinamide. A polymer is formed by the continued addition of ADP-ribose units to the initial residue.

The discovery of poly(ADP-ribose) and the elucidation of its structure were made independently in three laboratories (Chambon et al, 1966; Fujimura et al, 1967; Nishizuka et al, 1967). Shortly thereafter, the covalent association of poly(ADP-ribose) with chromatin proteins was demonstrated (Nishizuka et al, 1968). Subsequently, the main focus of research involved the identification of poly(ADP-ribosyl)ated proteins, the nature of the protein-(ADP-ribose) linkage, the metabolism and enzymology of poly(ADP-ribose) and the relationship of poly(ADP-ribose) synthesis with various cellular processes. These subjects have been covered in a large number of reviews (Sugimura, 1973; Hilz & Stone, 1976; Hayaishi & Ueda, 1977; Purnell et al, 1980; Hilz, 1981; Mandel et al, 1982; Pekala & Moss, 1983) and in an excellent treatise (Hayaishi & Ueda, 1982).

1. Poly(ADP-ribose): Structure, Properties and Metabolism

Poly(ADP-ribose) formation has been demonstrated in almost every eukaryotic system examined. The homopolymer

is composed of ADP-ribose [adenosine-5'-diphospho-5- β -D-ribose] joined by $\alpha(1''+2')$ glycosidic bonds (Miwa et al, 1977b; Ferro & Oppenheimer, 1978) and branches are formed through $\alpha(1''+3')$ linkages (Miwa et al, 1979). The in vivo presence of poly(ADP-ribose) has been verified by immunofluorescence (Ikai et al, 1980) and radioimmunoassays (Sakura et al, 1977). The in vivo existence of branching has also been demonstrated (Kanai et al, 1982; Juarez-Salinas et al, 1982). Natural antibodies to poly(ADP-ribose) are produced in patients with systemic lupus erythematosus and preliminary data suggest that the antigen is oligo(ADP-ribosyl)ated histone or free poly (ADP-ribose) (Kanai & Sugimura, 1982). Various chemical and immunological analyses indicate the presence of 5 to 500 nmoles of ADP-ribose per milligram of DNA (Niedergang & Mandel, 1982) with the bulk of the residues being derived from mono(ADP-ribosyl)ation (Hilz, 1981).

Poly(ADP-ribose) has a number of unique properties (Hilz & Stone, 1976) including resistance to deoxyribonucleases, ribonucleases, proteases and several phosphodiesterases. The pyrophosphate bond is cleaved by phosphodiesterases from snake venom (Chambon et al, 1966; Fujimura et al, 1967; Nishizuka et al, 1967), rat liver (Futai & Mizuno, 1967) and tobacco (Miwa et al, 1975). Digestion by snake venom phosphodiesterase yields three products which are separable by thin layer, paper or Dowex 1 chromatography (Shima et al, 1969). These products are 5'-AMP from

5', 5''-bis(phosphate)] and phosphoribosyl-isoADPribose [2'-(1''-riboseyl-2''-(1'''-riboseyl))adenosine-5',5'',5'''-tris-(phosphate)] from the branch points. The minimum number of ADP-ribose residues per poly(ADP-ribose) molecule is calculated as follows (Sugimura & Miwa, 1982):

$$\frac{[\text{AMP}] + [\text{isoADPR}] + [\text{pr-isoADPR}]}{[\text{AMP}] - [\text{pr-isoADPR}]}$$

The denominator signifies the number of poly(ADP-ribose) chains. Chain length has also been calculated by polyacrylamide gel electrophoresis (Tanaka et al, 1978; Adamietz et al, 1978a) and by chromatography on hydroxyapatite (Tanaka et al, 1977) and DEAE-cellulose (Kawaichi et al, 1981a). However, these methods do not correct for branching.

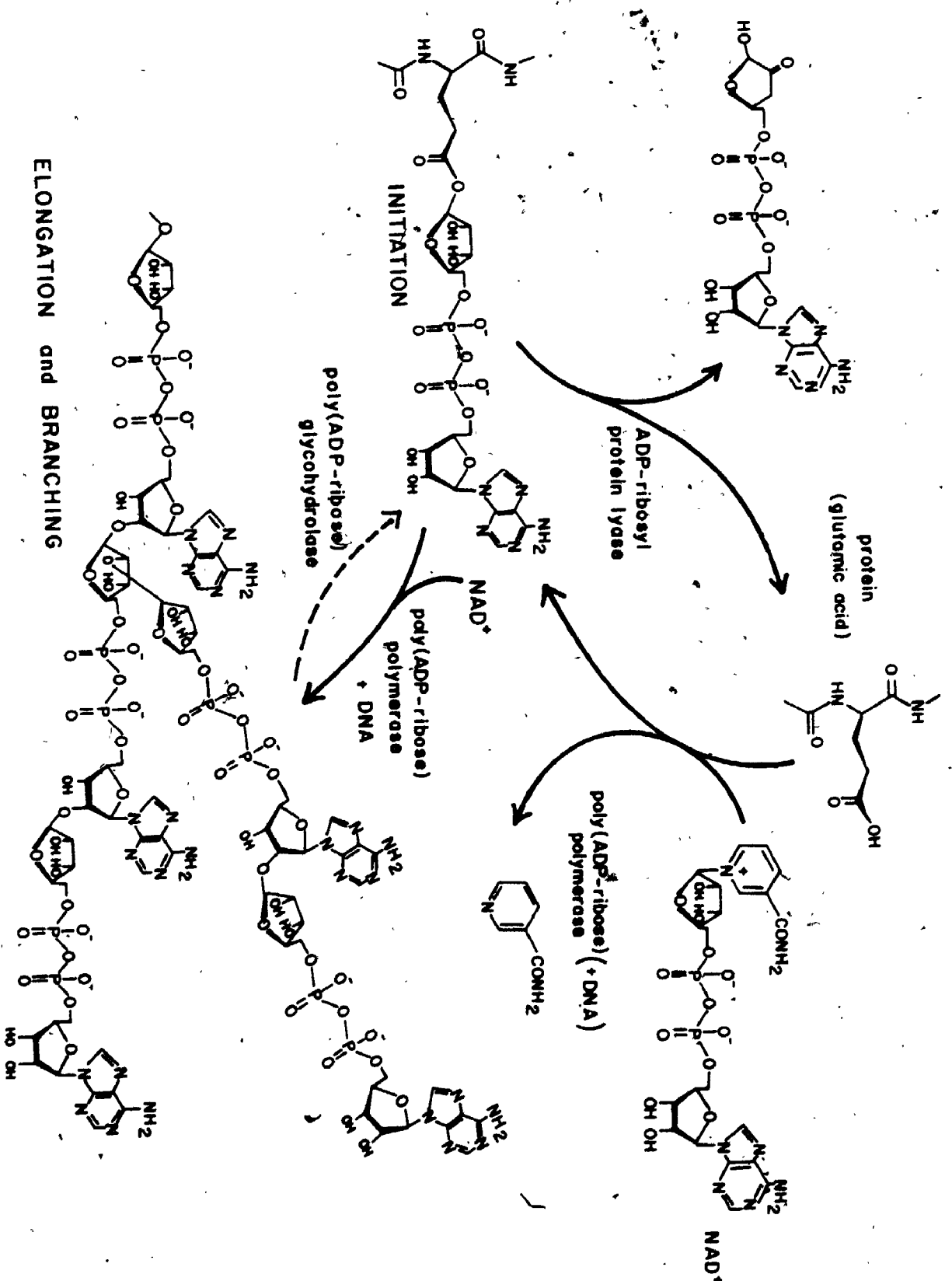
Two types of protein-(ADP-ribose) bonds have been identified (Adamietz & Hilz, 1976). All linkages are alkali-labile and the majority of the bonds are cleaved by neutral NH_2OH . The NH_2OH -sensitive bonds have been identified as ester links between the carboxyl group of the amino acid (usually glutamic acid) and the ribose residue (Riquelme et al, 1979; Burzio et al, 1979). Since NH_2OH -resistant bonds are found on modified proteins which have only ester bonds, it has been suggested that the resistant bonds are formed by isomerization of the ester bonds (Hilz, 1981). Formation of NH_2OH -resistant bonds by another enzyme has not been excluded and a linkage to arginine is also a possibility (Moss et al, 1983).

The metabolism of poly(ADP-ribose) involves three enzymes (refer to Fig. 1). Poly(ADP-ribose) polymerase [NAD:protein(ADP-ribose) ADP-ribosyltransferase] initiates polymer formation by attaching the initial ADP-ribose residue to a protein, elongates the polymer and inserts branches. These DNA-dependent reactions use the bond energy of the β -N-glycosidic nicotinamide-ribose linkage (-8.2 kcal/mol) as the driving force for the synthetic reaction. Poly(ADP-ribose) glycohydrolase (Miwa & Sugimura, 1982) degrades poly(ADP-ribose) exoglycosidically to yield ADP-ribose. The branch structures are removed by this enzyme but the ADP-ribose residue adjacent to the protein is not. ADP-ribosyl protein hydrolase (Oka et al, 1982) removes this final residue to yield AMP attached to a unique pentose sugar (Komura et al, 1983). The essential nature of this enzyme has recently been described (Williams et al, 1984). These three enzymes have been extensively purified (Ueda et al, 1982; Oka et al, 1982; Tavassoli et al, 1983; Oka et al, 1984).

Free poly(ADP-ribose) is not produced in large quantities by purified poly(ADP-ribose) polymerase under standard in vitro assay conditions (Yoshihara et al, 1977; Kawaichi et al, 1981b), although it is formed by certain extracts (Rickwood et al, 1977; Benjamin & Gill, 1980b). Poly(ADP-ribose) inhibits a deoxyribonuclease (Yamada et al, 1974), a chromatin-bound serine protease (Murachi, 1982) and a Mg^{2+} -dependent endonuclease (Tanigawa &

Figure 1

Poly(ADP-ribose): Structure and Metabolism. One of the proposed branch structures is represented.



ELONGATION and BRANCHING

Shimoyama, 1983). It is not known whether protein-bound poly(ADP-ribose) is effective as an inhibitor.

2. Poly(ADP-ribose) Polymerase

Poly(ADP-ribose) polymerase has been found in every eukaryotic species examined with the possible exceptions of Neurospora crassa and yeast, but it has never been found in prokaryotic organisms. The enzyme has been localized in the nucleus and is tightly associated with chromatin (Ueda et al, 1968; Hilz & Kittler, 1968). Within the chromatin, poly(ADP-ribose) polymerase is associated with the inter-nucleosomal DNA and appears to be located between the solenoidal helices of the nucleosomes although this latter point is still controversial (Butt et al, 1978).

Poly(ADP-ribose) polymerase has been purified from a variety of sources and extensive studies have determined the physical properties of the enzyme (Ueda et al, 1982). These studies have indicated slight species variation (Jongstra-Bilen et al, 1981; Holtlund et al, 1981). The enzyme is composed of a single polypeptide with a molecular weight of 110,000-130,000. Enzymatic activity is dependent on DNA (Yamada et al, 1971; Yoshihara, 1972) containing nicks or double-stranded breaks (Ohgushi et al, 1980; Benjamin & Gill, 1980a).

Poly(ADP-ribose) polymerase catalyzes the elongation of poly(ADP-ribose) by terminal addition. It is not known if the reaction is processive or distributive. Since

branching of poly(ADP-ribose) was found in systems using purified poly(ADP-ribose) polymerase, it has been concluded that this enzyme also catalyzes the branching reaction. Free ADP-ribose has been observed with a partially purified enzyme which implies it may act as an NADase under certain conditions (Ueda et al, 1975a).

Enzyme activity is affected by a number of components. Histones are activators of poly(ADP-ribose) polymerase activity under conditions of low histone to DNA ratios (Okavama et al, 1977) and serve as acceptors for poly(ADP-ribose) at high ratios (Kawaichi et al, 1980). Mg^{2+} modulates the effect of histones (Tanaka et al, 1979). Divalent cations, polyamines and thiol compounds are stimulative. Inhibition by N-ethylmaleimide and p-chloro-mercuribenzoic acid suggest a requirement for sulfhydryl groups. Both the temperature ($25^{\circ}C$) and the pH (8.0 to 8.5) optima of the calf thymus enzyme differ from the physiological norm. A large number of inhibitors, including analogues of NAD^{+} and nicotinamide, or purine and pyrimidine derivatives, have been identified (Ueda et al, 1982) and utilized in biological studies.

3. Acceptor Proteins

An extensive list of acceptor proteins for poly(ADP-ribose) has been compiled. These include histone H1 and various core histones (Nishizuka et al, 1968), histone H6 (Wong et al, 1977), the high mobility group (HMG) proteins

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(Reeves et al, 1981); trout protamines (Wong et al, 1977), protein A24 (Okayama & Hayaishi, 1978), SV40 T-antigen (Goldman et al, 1981), a Ca^{2+} , Mg^{2+} -dependent endonuclease (Yoshihara et al, 1974), a DNA topoisomerase (Ferro et al, 1983; Jongstra-Bilen et al, 1983) and poly(ADP-ribose) polymerase (Yoshihara et al, 1977). Of these, only the histones (Adamietz et al, 1978b; Ueda et al, 1975b; Ord & Stocken, 1977), SV40 T-antigen (Goldman et al, 1981) and the HMG proteins (Tanuma & Johnson, 1983) have been shown to be modified in vivo. It has been suggested that RNA polymerase I and DNA ligase II might be acceptors for poly(ADP-ribose) (Muller & Zahn, 1976; Creissen & Shall, 1982), but the initial observations have not been verified. There are also a host of unidentified proteins which appear to be modified by poly(ADP-ribose) (Malik et al, 1983; Surowy & Berger, 1983a; Faraone-Mennella et al, 1982; Kawashima & Izawa, 1981).

Studies of the in vivo acceptors for poly(ADP-ribose) have been severely handicapped due to the impermeability of the plasma membrane to NAD^+ . Because of these difficulties, isolated nuclei or permeabilized cells have been used for identifying poly(ADP-ribosyl)ated proteins. A major concern with these studies is the possibility of DNA damage, followed by activation of poly(ADP-ribose) polymerase causing artifactual poly(ADP-ribosylation) (Adamietz, 1982; Kreimeyer et al, 1984). The identification of poly(ADP-ribosyl)ated proteins is further

complicated by the lability of the protein-(ADP-ribose) bond to mild alkaline conditions and the increase in the apparent size and charge density of the modified protein. The use of various matrices to separate modified from unmodified proteins should help in the identification of the acceptors (Okayama et al, 1978; Malik et al, 1983).

The relationship of histone modification and chromatin structure has been a subject of primary interest. Histones H1 and H2b have been studied the most extensively and the modified amino acids have been identified (Riquelme et al, 1979; Burzio et al, 1979; Ogata et al, 1980a/b). In the case of Ca^{2+} , Mg^{2+} -endonuclease, poly(ADP-ribose) polymerase and DNA topoisomerase, modification by poly(ADP-ribose) inhibits enzymatic activity (Yoshihara et al, 1974; Ferro et al, 1983; Kawaichi et al, 1981b; Jongstra-Bilen et al, 1983). The activation of RNA polymerase II (Slattery et al, 1983) and DNA ligase II (Creissen & Shall, 1982) by poly(ADP-ribosylation) may occur via the modification of inhibiting proteins (Slattery et al, 1983; Ohashi et al, 1983). The modification of certain proteins depends on the state of the cell and different patterns of poly(ADP-ribosylation) have been noted in quiescent, growing and DNA damaged cells (Surowy & Berger, 1983a; Thi Man & Shall, 1982; Thraves & Smulson, 1982).

4. Biological Function

The function of poly(ADP-ribosylation) remains a

mystery despite the wealth of publications on this topic. The difficulty of identifying poly(ADP-ribosyl)ated proteins, as previously indicated, makes it virtually impossible to correlate their modification to a specific cell function. Similarly, the results of inhibitor studies are hard to interpret. This may be due to the questionable specificity of the inhibitors used in the various studies (Milam & Cleaver, 1984; Boorstein & Pardee, 1984). The development of variant cell lines has been attempted, but conclusive studies are lacking (Kidwell & Burdette, 1974; Nduka & Shall, 1980). Despite these uncertainties, enough data has accumulated to suggest a role for poly(ADP-ribosyl)ation in certain cellular processes.

4a. DNA Repair: The initial observations indicated that nuclease treatment of DNA stimulated poly(ADP-ribose) polymerase activity (Janakidevi & Koh, 1974; Miller, 1975) and that DNA damaging agents decreased NAD^+ pools (Roitt, 1956). These observations suggested that poly(ADP-ribose) was a component in DNA repair. A relationship of poly(ADP-ribosyl)ation and DNA repair has been demonstrated by the use of a series of inhibitors (Rankin et al, 1980). Interestingly, the increase in poly(ADP-ribose) polymerase activity and the concomitant decrease in NAD^+ concentration do not result in a net accumulation of poly(ADP-ribose), indicating a rapid turnover of the polymer (Wielkins et al, 1982).

Inhibitors of poly(ADP-ribose) polymerase retard the ligation of the repair patch (Durkacz et al, 1980b; James & Lehman, 1982) resulting in a net inhibition of excision repair (Durkacz et al, 1980b). While contrasting results have been obtained with terminally differentiated cells (Bohr & Klenow, 1981; Althaus et al, 1982b), most data suggest a role for poly(ADP-ribose) polymerase in the ligation step of excision repair. The observation that poly(ADP-ribosyl)ation activates DNA ligase has strengthened this viewpoint although the mechanism by which this activation occurs has not been defined. Poly(ADP-ribosyl)ation of DNA ligase has been described as one way by which ligase is activated (Creissen & Shall, 1982). It has also been observed that histones inhibit ligase activity and that the presence of poly(ADP-ribose) reverses this inhibition (Ohashi et al, 1983). Poly(ADP-ribosyl)ation may also function in DNA repair at a step unrelated to ligation (Waters et al, 1982; Durrant et al, 1981). The often-observed enhancement of DNA synthesis by poly(ADP-ribose) polymerase inhibitors (WieWkins et al, 1982; Durkacz et al, 1981; Cleaver et al, 1983; Sims et al, 1983) appears to be linked to the higher NAD^+ and ATP pools resulting from inhibition of poly(ADP-ribose) polymerase (Sims et al, 1983). It also appears that only excision repair of DNA damaged by alkylating agents requires poly(ADP-ribose) synthesis (James & Lehman, 1982; Nolan & Kidwell, 1982; Charles & Cleaver, 1982; Cleaver et al, 1983).

4b. Cell Growth: Numerous studies have implied a link between cell growth and poly(ADP-ribose) synthesis. An inverse relationship between DNA synthesis and poly(ADP-ribose) formation has been described on the basis of inhibitor studies (Burzio & Koide, 1970) and comparative activities of DNA and poly(ADP-ribose) polymerases in the cell (Miwa et al, 1973; Berger et al, 1978b). Other studies have questioned these relationships (Lehmann et al, 1974; Savard et al, 1981; Hilz & Kittler, 1971; Berger et al, 1978b). In particular, the inhibitor studies were faulted since the DNA polymerase α inhibitors caused an accumulation of DNA strand breaks which could activate poly(ADP-ribose) polymerase (Fram & Kufe, 1982). Despite these uncertainties, an involvement of poly(ADP-ribose) in some aspect of DNA synthesis appears likely. Poly(ADP-ribose) formation was shown to be cell cycle-specific (Miwa et al, 1973; Colyer et al, 1973; Kidwell & Mage, 1976; Berger et al, 1978a; Tanuma et al, 1978; Tanuma & Kanai, 1982). Poly(ADP-ribose) polymerase inhibitors caused a partial block in the G2 phase of the cell cycle (Kidwell & Burdette, 1974) as well as an extension of the S phase (Schwartz et al, 1983). These inhibitors also prevented the initiation of DNA synthesis in mitogen-stimulated lymphocytes (Perrella, 1982). Diadenosine tetraphosphate (Ap_4A), a ligand of DNA polymerase α and a possible signal for S phase, has been poly(ADP-ribo)sylated in vitro (Yoshihara & Tanaka, 1981). Although a link between cell

growth and poly(ADP-ribosyl)ation has been observed, a definitive role for poly(ADP-ribose) in cell growth has not yet been established.

4c. Transcription and Gene Expression: The role of poly(ADP-ribosyl)ation in transcription and gene expression is not clearly established, but several proteins involved in these processes are modified by poly(ADP-ribose). Poly(ADP-ribosyl)ation of RNA polymerase I (Muller & Zahn, 1976), protein A24 (Okayama & Hayaishi, 1978) and the HMG proteins (Tanuma & Johnson, 1983) has been demonstrated. Protein A24 is a repressor of ribosomal gene expression (Ballal & Busch, 1973) and the HMG proteins function in transcription. SV40 T-antigen, which is involved in both transcription and replication, is also poly(ADP-ribosyl)ated (Goldman et al, 1981). RNA polymerase II has been activated in vitro by poly(ADP-ribosyl)ation (Slattery et al, 1983). Related processes such as hnRNA particle formation (Kostka & Schweiger, 1982) and steroid hormone-induced changes in gene activity (Shimoyama et al, 1982) have been linked to poly(ADP-ribose). Although this evidence supports a role for poly(ADP-ribose) in transcription and gene expression, the significance of poly(ADP-ribosyl)ation in these processes has been questioned (Muller & Zahn, 1976; Althaus et al, 1982a; Walker & Pearson, 1981; Taniguchi et al, 1982).

4d. Differentiation: Recently there has been a flurry of interest in the possibility that poly(ADP-ribose) polymerase functions in cell differentiation (Williams & Johnstone, 1983). Various systems have been used for these studies including embryonic mesenchymal cells (Caplan & Rosenberg, 1975), Friend erythroleukemic cells (Tereda et al, 1979), intestinal epithelial cells (Porteus et al, 1982), human lymphocytes (Johnstone & Williams, 1982), adipocytes (Lewis et al, 1982), Xenopus laevis (Farzaneh & Pearson, 1978), cardiac muscle cells (Claycomb, 1976) and slime mould (Rickwood & Osman, 1979). In general, higher poly(ADP-ribose) levels were found during differentiation although contrasting results have been published as well (Williams et al, 1983). Furthermore, the differentiation processes were disturbed by inhibitors of poly(ADP-ribose) polymerase. These observations indicated a role for poly(ADP-ribose) in the initial stages of differentiation rather than in the later stages. Poly(ADP-ribose) might be involved in a joining reaction involving DNA which, according to a current model of differentiation, would occur during transposition of the genetic material (Johnstone & Williams, 1983).

4e. Chromatin Structure: The basic level of chromatin structure is the nucleosome which is composed of an octamer of four types of histones. The poly(ADP-ribosylation) of histones has been suggested as a mechanism for modulating

chromatin structure. The observation that poly(ADP-ribosyl)ated histones bound tighter to polynucleosomes (Poirier & Savard, 1978; Yoshihara et al, 1981) and the possible existence of a histone H1 dimer joined by a poly(ADP-ribose) chain has implied a role for poly(ADP-ribose) in chromatin condensation (Stone et al, 1977; Wong et al, 1983; Smulson et al, 1982). This suggestion has been supported by the immunofluorescent localization of both poly(ADP-ribose) and poly(ADP-ribose) polymerase in the heterochromatin region adjacent to the nuclear envelope (Ikai et al, 1982). In contrast, evidence for a role of poly(ADP-ribosyl)ation in chromatin relaxation has been published (Poirier et al, 1983). In this context, it has recently been shown that the histone H1 dimer does not exist (Aubin et al, 1982). Furthermore, poly(ADP-ribosyl)ated histones showed a decreased affinity for DNA (Burzio et al, 1980). Alterations in chromatin structure occur when histone H1 is replaced by histone H1°. Exchange of these proteins may be promoted by poly(ADP-ribosyl)ation since histone H1 is an acceptor of poly(ADP-ribose) while histone H1° is not (Poirier & Savard, 1978).

4f. Cell Transformation and Carcinogenesis: Poly(ADP-ribose) might be a component in cell transformation and carcinogenesis (Sugimura & Miwa, 1983). For example, transformed cells have higher levels of poly(ADP-ribose) (Ueda & Hayaishi, 1982; Miwa et al, 1977; Hilz & Kittler,

1971) and a correlation of altered poly(ADP-ribose) synthesis with aberrant cell differentiation has been hypothesized (Ueda & Hayaishi, 1982). Granulocytes which normally have no poly(ADP-ribose), show poly(ADP-ribose) synthetic activity in most types of leukemia (Ueda & Hayaishi, 1982). Poly(ADP-ribose) polymerase inhibitors are not mutagenic (Schwartz et al, 1983). These inhibitors have been shown to promote (Takahashi et al, 1982) and inhibit (Kun et al, 1983; Borek et al, 1984) chemical oncogenesis and may aid in the treatment of various neoplastic conditions (Smulson et al, 1977). Certain antineoplastic agents function by killing tumour cells and the cytotoxic effect of these agents is enhanced by poly(ADP-ribose) polymerase inhibitors, possibly by inhibiting DNA repair (Yamamoto & Okamoto, 1982).

4g. Mechanisms: While the exact function of poly(ADP-ribose) in the cell is not known, there are several mechanisms by which poly(ADP-ribosylation) might affect cell function. For example, the direct modification of a protein is known to alter enzymatic (Yoshihara et al, 1974; Ferro et al, 1983; Kawaichi et al, 1981; Jongstra-Bilen et al, 1983) and DNA-binding properties (Burzio et al, 1980; Poirier & Savard, 1978; Yoshihara et al, 1981). Enzymatic activity can also be affected by the modification of a nucleotide ligand (Le'John et al, 1975; Yoshihara & Tanaka, 1981; Tanaka et al, 1981) or another protein (Slattery et

al, 1983; Ohashi et al, 1983). A change in the chromatin structure via histone poly(ADP-ribosyl)ation may expose certain proteins or sections of DNA to different environments (Roberts et al, 1974). Also, the changes in NAD^+ and ATP pools by poly(ADP-ribosyl)ation (Rickwood & Osman, 1979; Sims et al, 1983) may affect various processes. A link between cell division, NAD^+ concentrations and poly(ADP-ribose) has been proposed (Ghani & Hollenberg, 1978) and NAD^+ levels may control the differentiation process (Caplan & Rosenberg, 1975). It is possible that poly(ADP-ribosyl)ation might function by all of these mechanisms.

5. Mono ADP-Ribosylation

Some ADP-ribose transferases from prokaryotic cells function as toxins by inhibiting essential cellular reactions in eukaryotes (Hayaishi & Ueda, 1977; Hilz, 1981; van Hevninger, 1980; Vaughan & Moss, 1981). Mono(ADP-ribose) transferases from eukaryotic cells are often masked by the high activity of poly(ADP-ribose) polymerase. The methodology used to study poly(ADP-ribosyl)ation rarely takes into account the presence of mono(ADP-ribosyl)ation. Mono(ADP-ribose) adducts are more numerous than poly(ADP-ribose) chains (Hilz, 1981). Many studies have implicated mono(ADP-ribosyl)ation in metabolite transport (Hofstetter et al, 1981; Hammerman et al, 1982), hormonal cell regulation (DeWolf et al, 1981; Vitti et al, 1982; Reilly et al, 1981), cell growth and differentiation (Hilz, 1981),

the cellular response to stress (Carlsson & Lazarides, 1983) and the control of histone phosphorylation (Tanigawa et al, 1983). The biological studies suggest that these mono(ADP-ribose) transferases may have a regulatory role (see above, ReBois et al, 1983) and enzymatic studies have identified several ADP-ribose transferases which are present in the cytosol, the nucleus and the plasma membrane (Tanigawa et al, 1984; DeWolf et al, 1981; Yost & Moss, 1983; Moss et al, 1980). Due to the similarities of poly(ADP-ribosylation) and mono(ADP-ribosylation), any conclusions on the function of poly(ADP-ribosylation) must eliminate the effects of mono(ADP-ribosylation).

MATERIALS AND METHODS

1. Materials

1a. Radioactives: [adenine-2,8-³H]-NAD⁺ (3.4 Ci/mmol) and [methyl-³H]-thymidine (6.7 Ci/mmol) were purchased from New England Nuclear and [carbonyl-¹⁴C]-NAD⁺ was bought from the Radiochemical Centre, Amersham.

1b. Chemicals: Chelex 100 and Bio-Gel HTP were from Bio-Rad, highly polymerized calf thymus DNA was from Sigma Chemical Co., Matrex gel red A was from Amicon Corp., blue Sepharose CL6B, Sephadex G-25 and Sephadex G-150 were from Pharmacia, Ultrogel Aca44 was from LKB, DEAE-cellulose and PEI-cellulose thin-layer chromatography sheets were from Brinkmann, while calf thymus lysine-rich and lyophilized histones were from Worthington Biochemicals. Ultrapure ammonium sulfate and Tris base were obtained from Swartz/Mann, while inorganic salts, glycerol and EDTA (all "AnalaR" grade) were supplied by British Drug Houses. Heavy metal-free dithiothreitol was from Calbiochem.

1c. Enzymes: Snake venom phosphodiesterase and DNase I were products of Worthington Biochemicals, catalase was supplied by Boehringer-Mannheim and poly(ADP-ribose) glycohydrolase, purified approximately 500-fold, was a gift from Dr. M. Tarnowka. The glycohydrolase was free of NADase

activity indicating the absence of NAD glycohydrolase and poly(ADP-ribose) polymerase activities.

2. Poly(ADP-ribose) Polymerase Activity

2a. Poly(ADP-ribose) Polymerase Assay: Activity was measured as the trichloroacetic acid precipitable radioactivity incorporated from [adenine-2,8-³H]-NAD⁺. Samples were incubated for 5 min at 25°C in assay buffer (0.14 ml) containing 100 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, 10% glycerol, 1.5 mM dithiothreitol, 100 μM [³H]-NAD⁺, 10 μg calf thymus DNA and 10 μg lyophilized histones. The reaction was terminated with trichloroacetic acid (20% final concentration) and bovine serum albumin (1 mg) was added. The precipitates were collected on glass-fibre filters, washed with 5% trichloroacetic acid and counted for radioactivity. This assay is referred to as the standard assay in the text.

2b. General Reaction Mixture: A variation of the standard assay was used in time course, kinetic and inhibition experiments. The reaction mixture contained 100 mM Tris-HCl pH 8.0, 1.5 mM dithiothreitol, 100 μM [³H]-NAD⁺, 70 μg/ml calf thymus DNA and indicated amounts of poly(ADP-ribose) polymerase. In the text, this assay will be indicated as the reaction mix. It was used in the kinetic and inhibitor studies performed which required conditions

and volumes different from those in the standard assay. Any changes in the above conditions will be given in the appropriate legends.

2c. Activity in Presence of Degrading Enzymes: In order to assay the polymerase in the presence of poly(ADP-ribose) glycohydrolase, a double-label assay was devised in which [carbonyl- ^{14}C]-NAD $^{+}$ (12 $\mu\text{Ci}/\text{ml}$) was included in the above reaction mixture and the polymerase was assayed by its nicotinamide release activity. The reactions were terminated with 50% ethanol for nicotinamide analysis. The sample was chromatographed on DEAE-cellulose sheets with 2 mM NH_4HCO_3 as a solvent. Nicotinamide spots ($R_f = 0.78$) were visualized by ultraviolet light, cut out and counted for radioactivity.

3. Poly(ADP-ribose) Polymerase Purification

3a. Buffers: The extraction buffer and buffers A to C contained 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 10 mM β -mercaptoethanol. Additional components were added to the following buffers: extraction buffer - 0.3 M NaCl, 50 mM NaHSO_3 , 0.5 mM dithiothreitol (DTT); buffer A - 0.2 M NaCl, 50 mM NaHSO_3 , 1 mM DTT; buffer B - buffer A with 10% glycerol; buffer C - 0.25 M KCl, 10% glycerol, 5 mM DTT. Buffer D contained 2 M KCl, 10% glycerol, 10 mM β -mercaptoethanol, 5 mM DTT and 1 mM potassium phosphate (pH 7.2).

3b. Enzyme Purification: Fresh calf thymus, maintained on ice until cleaned, was frozen at -70°C . All subsequent procedures were performed at $0-4^{\circ}\text{C}$. The frozen tissue (500g) was minced and homogenized (60s) in extraction buffer (1500 ml) using a Waring blender. The homogenate was centrifuged ($7500\times g$) for 15 min. Solid $(\text{NH}_4)_2\text{SO}_4$ was added to the supernatant to 30% saturation, stirred for 30 min and centrifuged ($10000\times g$) for 30 min. The supernatant was brought to 70% saturation with $(\text{NH}_4)_2\text{SO}_4$, stirred for 90 min and centrifuged ($10000\times g$) for 40 min. The precipitate was suspended in buffer A (300 ml) and desalted by passage through a Sephadex G-25 column (10x32 cm, 2500 ml). The preparation was applied to a DNA-cellulose (Alberts & Herrick, 1971) column (5x8.5 cm, 170 ml), washed with buffer B (500 ml) and eluted with a linear gradient of 0.2 M to 1.2 M NaCl (1200 ml) in buffer B. The active fractions were combined, loaded onto a Matrex gel red A column (3.2x6.3 cm, 50 ml), washed with buffer C (120 ml) and eluted with a gradient of 0.25 M to 1.25 M KCl (400 ml) in buffer C. The active fractions were combined, solid KCl added to 2.0 M (final concentration) and stirred for 30 min. The enzyme was applied to a hydroxyapatite column (1.5x4.5 cm, 8.0 ml) which was washed with buffer D (20 ml) and eluted with a gradient of 1 mM to 60 mM potassium phosphate (60 ml) in buffer D. The active fractions were pooled and stored at -20°C . This enzyme preparation was used for the bulk of the experiments described.

Optionally, a fraction of the hydroxyapatite pool was applied to an Ultrogel ACA44 column and eluted with buffer B at a rate of 10ml/hr. The active fractions were combined and the extent of purification determined.

An enzyme preparation obtained by a variation of this protocol was used for certain experiments. The DNA-cellulose pool was diluted 2.5-fold in buffer A (-NaCl) and applied to a blue Sepharose column. The column was washed with buffer A and eluted with a gradient of 0.2 M to 0.75 M NaCl, in buffer A. The hydroxyapatite pool was applied to a Sephadex G-150 and eluted with buffer B containing 1 M KCl. This protocol gave a lower yield than the method described above but an equivalent purification.

4. Metal Analysis

4a. Contamination Control: Several precautions were taken to reduce foreign metal contamination. Polyethylene plasticware which had been treated with 2 N HNO₃ (Thiers, 1957) was used for storing buffers and collecting enzyme samples. Chelex 100 (NH₄⁺ form) was used to prepare H₂O and buffer E (Poiesz et al, 1974). Buffer E, adjusted to pH 8.0 with NH₄OH, contained 0.2 M (NH₄)₂SO₄, 50 mM MOPS and 5 mM dithiothreitol.

4b. Column Fractionation: A Sephadex G-25 column (0.9x27 cm, 15 ml) was treated initially with 5 ml of 1 mM 1,10-

phenanthroline, washed with 100 ml H₂O and equilibrated with buffer E. A sample (0.3 mg) of purified poly(ADP-ribose) polymerase was mixed with Chelex 100 and then applied to the Sephadex column. Fractions (0.5 ml) were collected, enzymatic activity was measured by the standard assay and protein was determined colourimetrically. Metal analyses were performed by atomic absorption spectroscopy at Barringer Magenta Ltd., Toronto, Canada.

4c. Dialysis Conditions: Purified poly(ADP-ribose) polymerase was dialyzed for 5 hr against buffer E containing 1,10-phenanthroline and 50 mM Tris-HCl pH 8.0 or 50 mM potassium phosphate pH 6.0. This was followed by a further 8 hr dialysis involving two changes of buffer E containing Chelex 100 (10 g/250 ml buffer). Triton X-100 (0.5%) was present in all buffers to prevent the loss of protein by adsorption on the dialysis tubing. Dialyzed samples were analyzed for enzyme activity, protein and zinc content.

4d. Measurement of ⁶⁵Zn Exchange: Exchange reactions were performed at 4°C for 48 hr in 140 µl (total volume) of 50 mM Tris-HCl pH 8.0 or 50 mM potassium phosphate pH 6.0 (Chelex 100-treated) containing 0.5 µCi/ml carrier-free ⁶⁵Zn and 22 pmol poly(ADP-ribose) polymerase. Exchange was measured as the retention of radioactivity after filtration on Whatman GF/C filters and washing with 4.0 ml cold buffer containing 50 mM Tris-HCl pH 8.0, 0.5 mM EDTA.

5. DNA Preparations

5a. Plasmid Preparation: ColE1 plasmid was purified from Escherichia coli JC411(ColE1) by the method of Birnboim & Doly (1979) modified for larger amounts of cells. Cells were grown in M9 minimal media to an optical density (600 nm) of 0.7-0.8 at which time chloramphenicol (200 μ g/ml) was added. Plasmid was labelled with 3.75 μ Ci/ml [3 H]-thymidine in the presence of 75 μ g/ml deoxyadenosine added 30 min after chloramphenicol. Cells were incubated for a further 18 hr, harvested by centrifugation, washed in phosphate-buffered saline and suspended in a buffer containing 25 mM Tris-HCl pH 8.0, 10 mM EDTA, 20% glucose and 2 mg/ml lysozyme. After 30 min on ice, 2 volumes of denaturation buffer (0.2 N NaOH, 1% SDS) was added. Tubes were maintained at 4°C with continuous agitation until the solution became transparent. The pH was neutralized with 1 volume of 3 M sodium acetate pH 4.8 and left on ice for 50 min. The precipitate was removed by centrifugation (12000xg for 15 min) and the plasmid DNA precipitated from the supernatant at -70°C with 4 volumes of 95% ethanol. The DNA was collected by centrifugation, re-precipitated with ethanol and suspended in a buffer containing 50 mM Tris-HCl pH 8.0, 100 mM sodium acetate pH 8.0, 1 mM EDTA. Relative amounts of supercoiled (form I) and relaxed (form II) circular DNA were determined by densitometry of the DNA after agarose gel electrophoresis.

5b. Enrichment for Supercoiled DNA: Since the relative proportion of form I to form II DNA varied with each preparation, a simple method for enhancing the amount of form I DNA was developed. The plasmid (0.6 ml) was reversibly denatured through a 10 sec exposure to 5 ml of buffer at pH 12.1 containing 0.9 M NaCl, 0.1 M K_2HPO_4 , 25 mM EDTA (Braun, 1981). The solution was neutralized with HCl and extracted with an equal volume of phenol containing 0.5 M NaCl, 1 mg/ml 8-hydroxyquinoline (McMaster et al, 1980). The phenol phase was re-extracted with water, the aqueous phases combined and extensively dialyzed against water. The DNA was recovered by precipitation at $-20^\circ C$ with 0.2 volumes of 3 M sodium acetate pH 4.8 and 4 volumes of 95% ethanol.

5c. Nuclease Treatment: Calf thymus or ColE1 plasmid DNA (0.6 $\mu g/ml$) was incubated in buffer (0.25 ml) containing 30 mM Tris-HCl pH 8.0, 0.5 mM EDTA, 10 mM $MgCl_2$, 0.05 mg/ml crystalline bovine serum albumin and varying amounts of pancreatic DNase I at $25^\circ C$ (Benjamin & Gill, 1980b). Samples (20 μl) were removed at various times and placed into tubes containing 0.1 ml reaction mix including 15 mM EDTA. Polymerase (30 pmol/ml) was added and activity was measured with these samples replacing added DNA.

5d. DNA Solubilization Assay: The degradation of [3H]-Col E1 DNA by various compounds was measured in reaction mix

(pH 6.0) minus NAD^+ and calf thymus DNA. After incubation for 20 min at 25°C , cold trichloroacetic acid (10% final concentration) was added, undegraded DNA was removed by centrifugation ($10000\times g$) for 10 min and the supernatant was counted for radioactivity.

6. Poly(ADP-ribose)

6a. Preparation and Purification: A large scale version of the reaction mixture (130 ml) containing 0.1 M Tris-HCl pH 8.0, 10 mM MgCl_2 , 1.5 mM dithiothreitol, 400 μM NAD^+ , 75 $\mu\text{g/ml}$ DNA, 40 $\mu\text{g/ml}$ histones, 20% ethylene glycol was incubated with 10 ml calf thymus 0.3 M NaCl extract for 60 min at 25°C . The reaction was stopped by the addition of 0.2 volumes of 3 M sodium acetate pH 4.8 and 2 volumes of 95% ethanol. After 18 hr at -20°C , the precipitate was collected by centrifugation, incubated in 0.1 N NaOH at 25°C for 60 min and neutralized with HCl. The solution was adjusted to 0.25% SDS, 0.075 M NaCl and 0.025 M Tris-HCl pH 8.0. This was incubated 4 hr at 25°C with pronase (1 mg/ml) followed by extraction with 0.8 volumes of water-saturated phenol. The phenol phase was extracted twice with a buffer containing 0.175 M NaCl, 0.05 M Tris-HCl pH 8.0. The aqueous phases were pooled and precipitated with ethanol (as above). The precipitate was suspended in a buffer (0.05 M Tris-HCl pH 8.0) containing 5 mM MgCl_2 and DNase I (2 mg/ml), incubated 4 hr at 25°C and extracted

with phenol. The pooled aqueous phases were mixed with 4 volumes of 50 mM sodium phosphate pH 6.8 and applied to a hydroxyapatite column (bed volume of 10 ml). The column was washed with 30 volumes of 0.1 M sodium phosphate pH 6.8 and eluted with a linear gradient of 0.1 to 0.5 M phosphate (20 column volumes). Collected fractions were examined by optical density at 260 nm and peak fractions were pooled, lyophilized and extensively dialyzed against water. Typical preparations yielded 0.25 to 1.0 mg poly(ADP-ribose).

6b. Quantitation: Estimates of poly(ADP-ribose) concentrations were made by optical density at 260 nm. Quantitative measurements were made by a fluorometric assay (Niedergang et al, 1978). ADP-ribose was the standard.

6c. Chain Length Analysis: Samples were digested with snake venom phosphodiesterase according to the method of Kawaichi et al (1980b). Digests were chromatographed on PEI-cellulose sheets in a solvent of 2 M acetic acid/0.25 M LiCl. Each strip was sliced into 1 cm sections and examined for radioactivity.

7. DNA-Protein Interactions

7a. Sedimentation Analysis: The samples (0.2 ml) were centrifuged in 5-20% neutral sucrose gradients containing

0.1 mM MgCl₂, 5 mM dithiothreitol, and 10 mM β-mercaptoethanol in an SW50.1 Beckman rotor at 45000 rev/min for 150 min at 10°C (Sakibara & Tomizawa, 1974). Fractions were collected from the bottom of the tube and analyzed as indicated in the text.

7b. Filter Binding Assay: The binding of [³H]-ColE1 plasmid DNA to poly(ADP-ribose) polymerase was measured as described by Ohgushi et al (1980). Aliquots of enzyme (1.4 μg) were incubated in 0.13 ml buffer F (50 mM potassium phosphate pH 6.0, 10 mM MgCl₂, 10 mM β-mercaptoethanol) at 25°C with various inhibitors. [³H]-ColE1 DNA (10 μg) was added, incubation continued for 1 min and 1.0 ml cold buffer F was added. The solution was filtered on Whatman GF/C filters pre-coated with 1 mg bovine serum albumin. The filters were washed twice with 2 ml buffer F, dried and counted for radioactivity. Less than 1% of the DNA was bound in the absence of protein.

8. Analytical Procedures

8a. Basic-SDS/Polyacrylamide Gel Electrophoresis: Samples were precipitated with 20% trichloroacetic acid, washed with acetone, suspended in 40 μl sample buffer (0.0625 M Tris-HCl, 10% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.001% bromphenol blue) and heated for 3 min at 100°C. The samples were run with a current of 30 mA at 4°C in a slab

gel of 7.5% polyacrylamide (29:1 acrylamide/bisacrylamide) with a 5% stacking gel according to the method of Laemmli (1970). The gels were stained with 0.125% Coomassie Blue in 50% methanol/10% acetic acid and destained in 20% methanol/10% acetic acid.

8b. Neutral-SDS/Polyacrylamide Gel Electrophoresis: Sample precipitates were resuspended in 20 μ l 50 mM potassium phosphate, pH 7.2, mixed with 20 μ l of sample buffer containing 0.15 M Tris/PO₄, pH 5.5, 20% glycerol, 2% SDS, 10% β -mercaptoethanol, 0.006% bromphenol blue and heated at 100°C for 3 min. Slab gel electrophoresis of the samples was performed using the neutral discontinuous anionic system of Williams and Reisfeld (1964), with slight modifications. The stacking gel contained 3% acrylamide (29:1 acrylamide/bisacrylamide), 0.06 M Tris/PO₄, pH 5.5, 1% SDS, 0.4% Temed and 0.06% ammonium persulfate. The separating gel had 5% acrylamide, 0.07 M Tris/PO₄, pH 7.5, 1% SDS, 0.15% Temed and 0.07% ammonium persulfate. The electrophoresis buffer (pH 7.0) contained 0.03 M barbital, 0.008 M Tris and 1% SDS. Electrophoresis was conducted for 4 hr at 20 mA. Gels were stained as described above or fluorographed with Kodak X-Omat AR5 film after enhancement in sodium salicylate (Chamberlain, 1979).

8c. Agarose Gel Electrophoresis: Samples were mixed with sample buffer (7 M urea, 50% sucrose, 50 mM EDTA, 2.5% SDS,

0.01% bromphenol blue) in a ratio of 2:1 (sample/buffer). The DNA was separated over 18 hr at 35 mA in 0.8% agarose at pH 8.2 in a buffer containing 0.04 M Tris, 0.02 M sodium acetate, 1 mM EDTA. The gel was stained for 60 min in 1 μ g/ml ethidium bromide and the DNA visualized by direct illumination with ultraviolet light. Records were kept by photographing the gel with Polaroid type 665 film for 25 sec.

8d. DNA Quantitation: DNA concentrations were estimated spectrophotometrically at 260 nm (50 μ g/ml DNA = 1.0 optical density unit). DNA was also measured by fluorescence assays using ethidium bromide (Morgan et al, 1979).

8e. Protein Determinations: Protein content was routinely monitored by absorbance at 280 nm. Protein quantitation was performed by a fluorescence assay (Gagerman, 1980) or colourimetrically (Lowry et al, 1951) after precipitation with trichloroacetic acid (Peterson, 1977). Bovine serum albumin was used as the standard.

9. Data Presentation: All experiments described in the text were repeated at least three times. Figures and tables represent the results of a typical experiment. Averaging and other mathematical manipulations of the data will be indicated in the legends.

RESULTS

1. Poly(ADP-ribose) Polymerase Purification

Poly(ADP-ribose) polymerase has been purified to near homogeneity from a variety of sources (Okayama et al, 1977; Mandel et al, 1977; Tsopanakis et al, 1978; Kristensen & Holtlund, 1978; Yoshihara et al, 1978; Ito et al, 1979; Jump & Smulson, 1980; Petzold et al, 1981; Carter & Berger, 1982) and studied intensively in vitro. We have purified poly(ADP-ribose) polymerase from calf thymus using a method similar to one described by Yoshihara et al (1978). The procedure used here differed in that a Matrex gel red A column was used after chromatography on DNA-cellulose and a gel filtration step was omitted after hydroxyapatite chromatography. After the hydroxyapatite step, the enzyme was freed from endogenous DNA and purified 1500-fold with a 14% yield (Table I). The yield has varied from 10% to 25%. Poly(ADP-ribose) polymerase is eluted from red A agarose at a higher salt concentration (1 M NaCl) than from blue Sepharose (0.3 M NaCl), resulting in improved purification and higher yield. SDS/polyacrylamide gel electrophoresis (Fig. 2) showed a major polypeptide with an apparent molecular weight of 114,000 (97%) and minor bands of 66,000 (2%), 75,000 (<1%) and 43,000 (<1%). The Mr 75,000 band may be a degradation product of the major polypeptide (Surowy & Berger, 1983b; Holtlund et al, 1983). With increased storage times, the amount of Mr 75,000 polypeptide

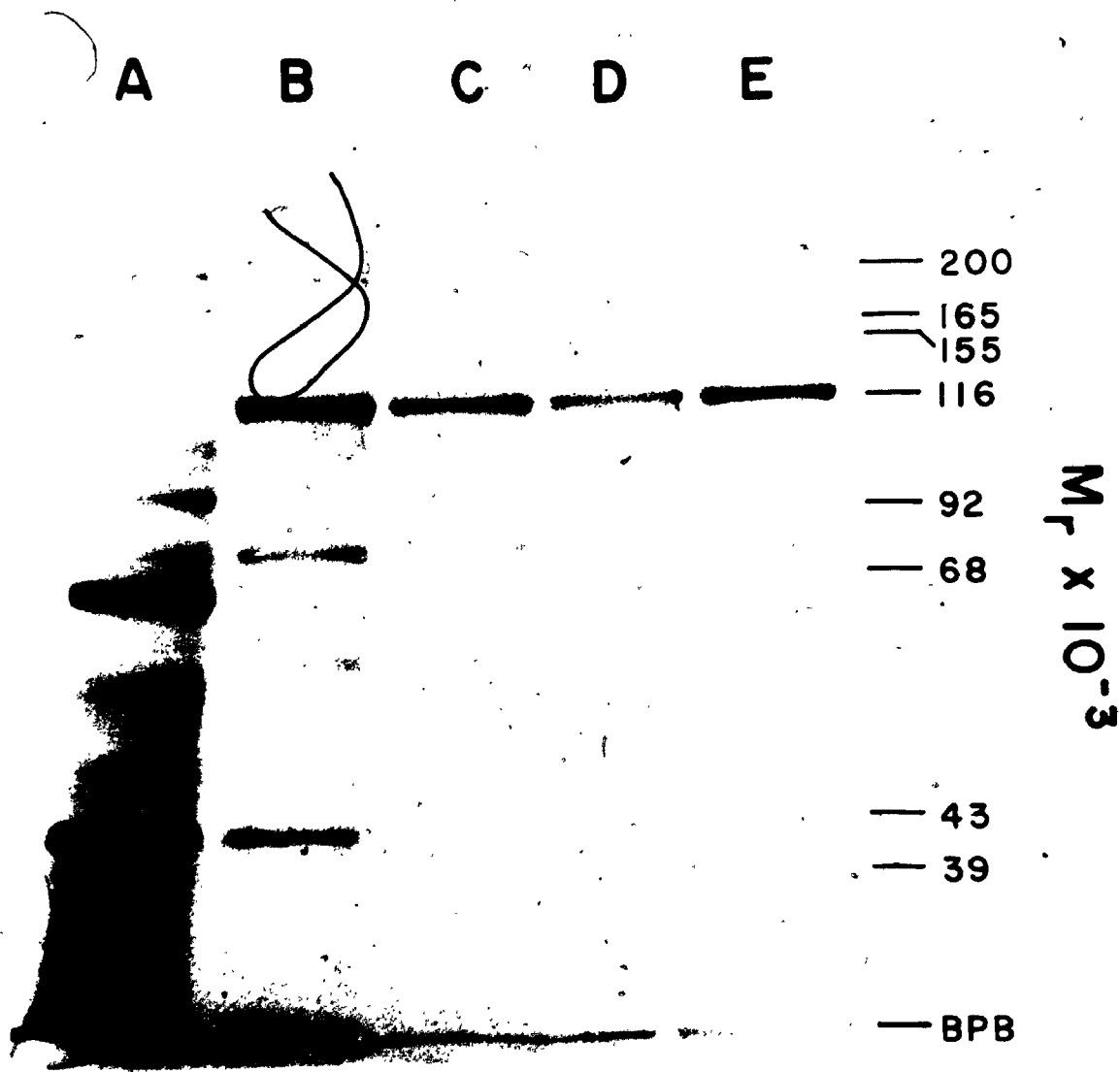
Table I

Purification of Poly(ADP-ribose) Polymerase. Activity was measured by the standard assay and protein determined colourimetrically. DNA dependence was calculated as activity (-DNA) / activity (+DNA).

FRACTION	TOTAL		SPECIFIC		YIELD	PURIFICATION	DNA DEPENDENCE
	PROTEIN	ACTIVITY	ACTIVITY	ACTIVITY			
	mg	nmol/min	nmol/min/mg	%	-fold	%	
extract	24000	60000	2.5	100	1.0	113	
(NH ₄) ₂ SO ₄	14300	56000	5.3	93	2.1	20	
DNA-cellulose	174	21170	112	35	49	20	
red-agarose	15.5	16160	1040	27	417	5	
hydroxyapatite	2.3	8590	3730	14	1490	0.1	

Figure 2

Electrophoretic Analysis of Stages of Poly(ADP-ribose) Polymerase Purification. Samples were precipitated with 20% trichloroacetic acid, rinsed with acetone and suspended in sample buffer. They were subjected to basic-SDS/polyacrylamide gel electrophoresis (7.5% polyacrylamide) and protein bands visualized by staining in Coomassie blue. Pooled samples include: extract (A), DNA-cellulose (B), red-agarose (C), hydroxyapatite (D) and Ultrogel Aca44 (E). Molecular weights standards included myosin (Mr 200000), RNA polymerase (Mr 165000, 155000, 39000), β -galactosidase (Mr 116000), phosphorylase b (Mr 94000) bovine serum albumin (Mr 68000) and ovalbumin (Mr 43000). BPB is bromophenol blue.



increased, especially at low protein concentrations. Gel filtration on Ultrogel AcA44 removed these contaminants (Fig.2) giving a slightly improved purification (up to 1.1-fold) but with a loss of 2/3 of the activity (data not shown). The latter consideration led us to use the hydroxyapatite fraction since this preparation was comparable in purity to other enzyme preparations (Okayama et al, 1977; Kristensen & Holtlund, 1978; Yoshihara et al, 1978; Petzold et al, 1981; Carter & Berger, 1982).

Enzyme activity required the addition of DNA while histones further stimulated the activity. Optimal activity occurred in the presence of $MgCl_2$ and dithiothreitol (although they were not absolutely required). Prolonged incubation (>5 min) in the absence of dithiothreitol resulted in a rapid decrease in activity. Mg^{2+} (10 mM optimum) could be replaced by other cations including Mn^{2+} (0.1 mM), Ca^{2+} (1 mM), Ba^{2+} (10 mM), Sr^{2+} (10 mM) (data not shown). The purified enzyme was maintained for several months in buffer D at $-20^\circ C$ without loss of activity.

2. Poly(ADP-ribose) Polymerase, a Zinc Metalloenzyme

It has been clearly demonstrated that the activation of polymerase requires DNA containing nicks or double-stranded breaks (Ohgushi et al, 1980; Benjamin & Gill, 1980b). Although the DNA structures which activate the enzyme have been identified (Benjamin & Gill, 1980b; Yoshihara & Kamiya, 1982), the basis for the DNA require-

ment is not understood.

Zinc has a functional role in a variety of DNA-dependent reactions (Auld, 1979; Valle & Falchuk, 1981; Coleman, 1983). Prokaryotic and eukaryotic RNA polymerases (Scrutton et al, 1971; Coleman, 1974; Auld et al, 1976; Lattke & Weser, 1976), terminal deoxynucleotide transferase (Chang & Bollum, 1970), restriction enzymes (Barton et al, 1982) and possibly DNA polymerases (Slater et al, 1971; Walton et al, 1982; Ferrin et al, 1983) are zinc metalloenzymes in which the metal is required for the interaction of the enzymes with DNA (Coleman, 1983; Chang & Bollum, 1970; Mildvan & Loeb, 1979). The results described below show that poly(ADP-ribose) polymerase is also a zinc metalloenzyme and that zinc appears to be involved in the interaction of the enzyme and DNA.

2a. Inhibition by Metal Chelators: The metal chelator, 1,10-phenanthroline, inhibits poly (ADP-ribose) polymerase at $\text{pH} < 8$ (Table II). Because of this pH dependence, all analyses involving chelators were performed in potassium phosphate buffer (pH 6.0). Inhibition was both time- and concentration-dependent (Fig! 3). Chelation of Mg^{2+} by 1,10-phenanthroline does not explain the inhibition since Mg^{2+} was not absolutely required for activity and was present in excess over inhibitor. Histones had no effect on the inhibition, while decreasing the incubation temperature slowed the rate of inhibition (data not

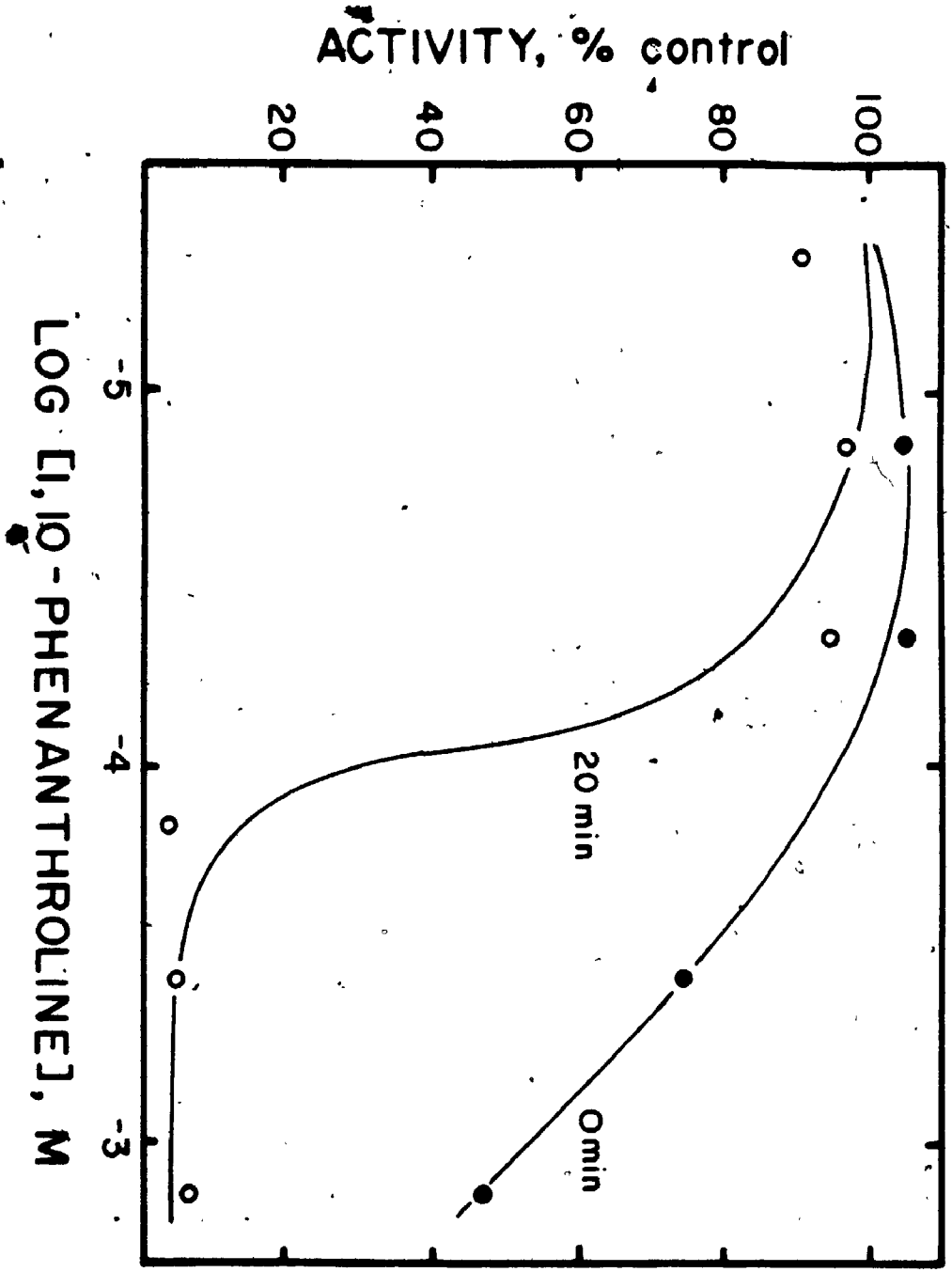
Table II

pH Dependence of Poly(ADP-ribose) Polymerase Inhibition by 1,10-Phenanthroline. The enzyme (10 pmol/ml) was incubated with 1 mM 1,10-phenanthroline (oP) for 20 min at 25°C in reaction mix (-NAD⁺, -DNA). Activity was determined by a 5 min incubation after the addition of [³H]-NAD⁺ (0.1 mM) and DNA (10 µg). Buffering agents used to maintain the pH were potassium phosphate (pH 5-6) and Tris-HCl (pH 7-10).

pH	ACTIVITY (nmol/min/mg)		
	-oP	+oP	+oP/-oP
5	12.1	0.6	5%
6	44.6	4.3	10%
7	37.7	10.3	27%
8	45.6	64.2	141%
9	28.8	38.7	112%
10	37.8	43.4	114%

Figure 3

Inhibition of Poly(ADP-ribose) Polymerase by 1,10-Phenanthroline. Enzyme (10 pmol/ml) was incubated for 0 min (●) or 20 min (○) at 25°C with various amounts of 1,10-phenanthroline in reaction mix (pH 6.0, -NAD⁺, -DNA). The reaction was initiated with the addition of [³H]-NAD⁺ (0.1 mM) and DNA (10 μg). The formation of poly(ADP-ribose) was measured under standard conditions.



shown). Most of the other chelating agents were non-inhibiting except for 2,2'-bipyridine and dithizone (Table III). Treatment of the enzyme with Chelex 100, CN^- , cysteine and imidazole occasionally gave a slight increase in activity above the control values. This may have been a consequence of the removal of inhibiting metal ions (Redetzki & Nowinski, 1957). 1,7-Phenanthroline, the non-chelating isomer of 1,10-phenanthroline, and 1,10-phenanthroline complexed with Co^{2+} showed little inhibition (Table III) indicating a chelation requirement.

Since poly(ADP-ribose) polymerase requires DNA for activity, the H_2O_2 -dependent degradation of DNA by 1,10-phenanthroline (Sigman et al, 1979) could explain the observed inhibition. We observed this degradation of DNA under our experimental conditions in the absence of poly(ADP-ribose) polymerase (Table IV). Catalase prevented this degradation (Table IV; Marshall et al, 1981) but did not prevent the inhibition of poly(ADP-ribose) polymerase (Table IV), suggesting that the inhibition by 1,10-phenanthroline was not the result of DNA degradation.

2b. Metal Identification: The purified poly(ADP-ribose) polymerase preparation was treated with Chelex 100, fractionated on a Sephadex G-25 column to remove metal contaminants and subsequently sent for metal analysis (Barringer-Magenta Ltd., Toronto). These data indicated the presence of approximately one μ -atom of zinc per mole

Table III

Effect of Metal Binding Agents on Poly(ADP-ribose) Polymerase Activity. Enzyme (10 pmol/ml) was incubated for 20 min at 25°C with various compounds in the reaction mix (pH 6.0, -NAD⁺, -DNA). Activity was measured by a 5 min incubation after addition of [³H]-NAD⁺ (0.1 mM) and DNA (10 μg). DTPA is diethylenetriamine pentaacetic acid.

COMPOUND	CONCENTRATION		INHIBITION
		mM	
1,10-phenanthroline		0.1	33
		0.5	97
1,7-phenanthroline		0.5	6
EDTA		0.5	16
quinoline-8-sulfate		0.5	12
DTPA		0.5	8
CN ⁻		2.0	0
2,2'-bipyridine		0.2	36
		1.0	85
neocuprone		0.2	7
		1.0	29
cysteine		1.0	0
dithizone		0.1	37
		0.25	55
		1.0	62
imidazole		1.0	0
Chelex 100		(0.14g/ml)	0
CoCl ₂		0.28	15
1,10-phenanthroline		1.0	97
CoCl ₂ + 1,10-phenanthroline		0.28+1.0	24

Table IV

Effect of DNA Degradation by 1,10-Phenanthroline on the Activity of Poly(ADP-ribose) Polymerase. DNA degradation was measured as the trichloroacetic acid solubilization of 10 μg [^3H]-ColE1 DNA after incubation for 20 min at 25° in reaction mix (pH 6.0, -NAD $^+$, -polymerase) containing 0.5 mM 1,10-phenanthroline. Poly(ADP-ribose) polymerase activity was determined in identically treated samples (+polymerase, -DNA) by incubating for 5 min after addition of [^3H]-NAD $^+$ (0.1 mM) and DNA (10 μg). Catalase (10 $\mu\text{g}/\text{ml}$) was added with the DNA.

CONDITIONS	DNA	
	SOLUBILIZED	INHIBITION
	%	%
no additions	0	0
1,10-phenanthroline	95	96
catalase	3	5
1,10-phenanthroline + catalase	5	98

of protein (Fig. 4, Table V). Similar results were obtained with another independently prepared sample which had not been treated with Chelex 100 (Table V). The zinc in the enzyme was available to the solvents at $\text{pH} < 8$ since the exchange with ^{65}Zn only occurred at $\text{pH} 6.0$. This exchange was specific for zinc since neither Mg^{2+} nor Cd^{2+} greatly affected the reaction (Table VI).

2c. Role of Metal: Dialysis of poly(ADP-ribose) polymerase under a variety of conditions indicated that a decrease in enzyme activity was associated with a corresponding decrease in the zinc content of the enzyme (Table VII). These observations are consistent with the previous inhibition studies (Table II) and indicate a role of zinc in enzyme activity. While the exposure of the enzyme to 1,10-phenanthroline at $\text{pH} 8.0$ did not lead to loss of activity (Table II), prolonged dialysis against a buffer ($\text{pH} 8.0$) containing the chelating agent and Triton X-100 did lead to a partial loss of activity and a corresponding reduction in zinc content.

Initial rate kinetics showed 1,10-phenanthroline was non-competitive with NAD^+ (Fig. 5A) and competitive with DNA (Fig. 5B) suggesting the metal was involved in the interaction of DNA with poly(ADP-ribose) polymerase. Binding of DNA to the enzyme is necessary for activity but 1,10-phenanthroline had no effect on DNA binding at inhibitory concentrations (Fig. 6).

Figure 4

Zinc Content of Poly(ADP-ribose) Polymerase.

Poly(ADP-ribose) polymerase (300 μ g) was applied to a Sephadex G-25 column after treatment with Chelex 100 and the fractions analyzed for activity (●), protein (○) and zinc (■). All data points are averages of duplicate assays.

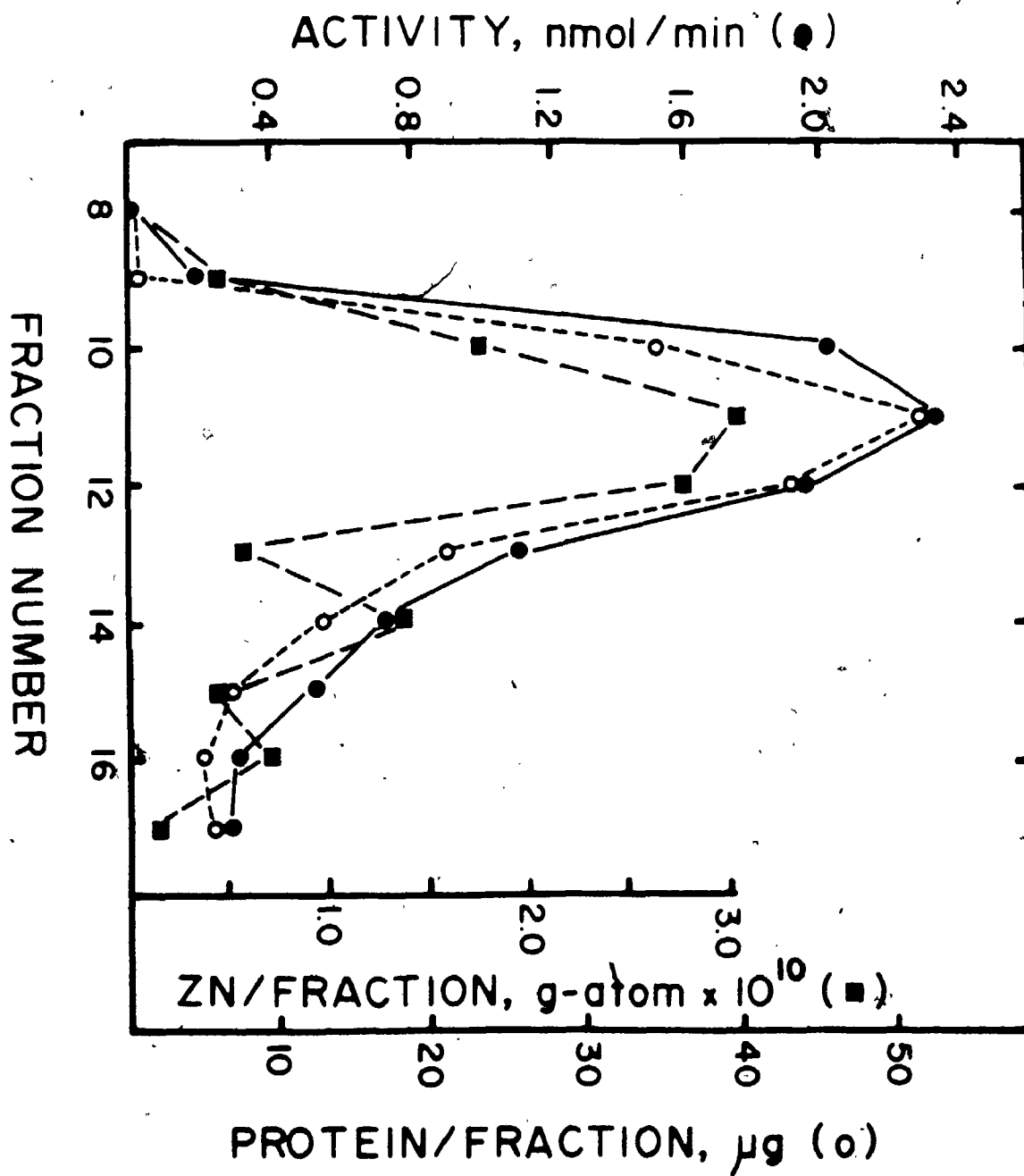


Table V

Metal Content of Poly(ADP-ribose) Polymerase. The enzyme was fractionated on Sephadex G-25 and fractions were analyzed for various metals. Zinc determinations were based on the averages of the four peak fractions. In I, 250 μ g of enzyme was analyzed without Chelex 100 treatment; in II, 300 μ g of a different enzyme preparation was treated with Chelex 100.

METAL	MOL/MOL PROTEIN	
	I	II
Co	<0.05	<0.05
Cr	<0.05	<0.05
Cu	0.07	0.07
Fe	0.12	0.03
Mo	<0.12	<0.06
Ni	0.19	0.06
Se	<0.14	0.10
V	<0.50	<0.17
Zn	1.11	0.78

Table VI

Exchange of ^{65}Zn with Poly(ADP-ribose) Polymerase Bound Zinc. Exchange was measured as the percentage of the total radioactivity in the reaction vessel retained on glass-fibre filters after a 48 hr incubation (4°C) of polymerase (22 pmol) with ^{65}Zn (0.5 $\mu\text{Ci/ml}$).

CONDITIONS	% EXCHANGE
pH8	1
pH6	30
pH6 + ZnCl ₂ (0.09mM)	7
pH6 + MgCl ₂ (0.09mM)	23
pH6 + CdCl ₂ (0.09mM)	26

Table VII

Correlation of Poly(ADP-ribose) Polymerase Activity and Zinc Content. Enzyme (130 μ g) was dialyzed for 5 hr against the conditions indicated followed by an 8 hr dialysis at pH 8.0 with two changes of buffer. Activity, protein and zinc content of the samples were then determined. The control activity (pH 8.0) represents 80% of the activity of the undialyzed enzyme. Zinc content varied up to 0.2×10^{-10} g-atoms in duplicate analyses.

CONDITIONS	ACTIVITY	PROTEIN	ZINC	ZINC/PROTEIN
	%	μ g	g-atoms	g-atoms/mole
pH8	100	114	9.5×10^{-10}	0.95
pH8 + oP	45	125	4.9×10^{-10}	0.45
pH6	76	114	7.4×10^{-10}	0.74
pH6 + oP	2	120	0.2×10^{-10}	0.02

Figure 5

Effect of 1,10-Phenanthroline on the Initial Rate Kinetics of Poly(ADP-ribose) Polymerase. Activity was measured in the absence (●) or presence (○) of 1,10-phenanthroline (1.4 mM) in reaction mix (pH 6.0) without pre-incubation. (A) Kinetics with NAD⁺, (B) kinetics with DNA. Data points are averages of duplicate assays and the slopes were determined by least squares regression.

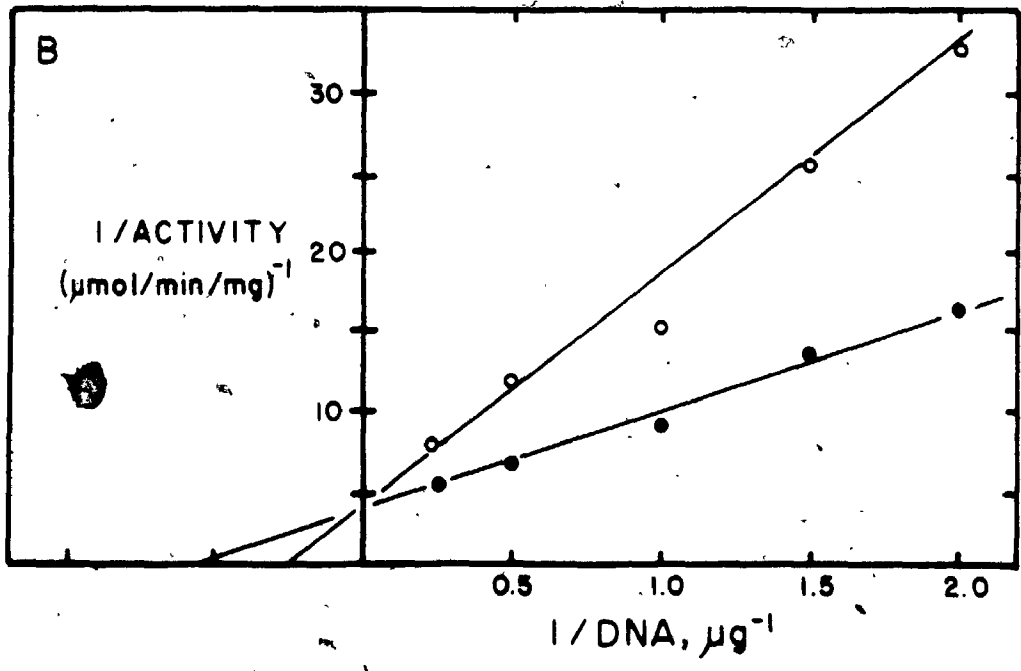
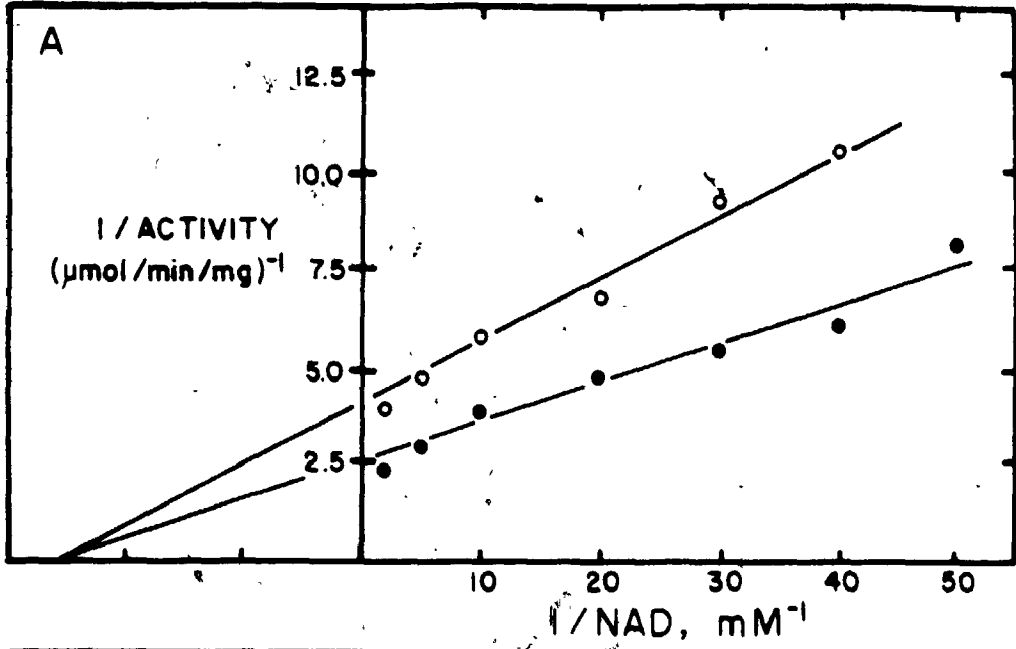
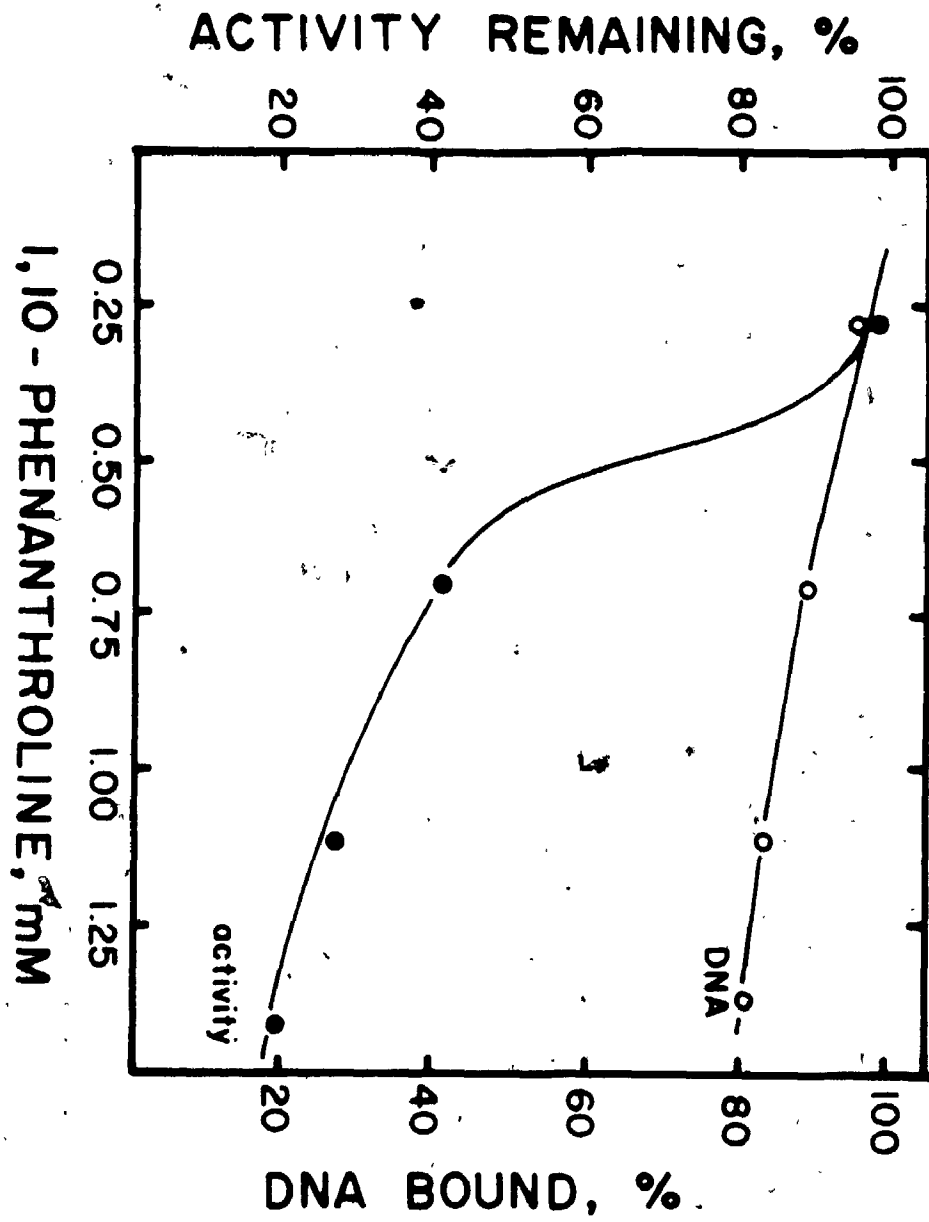


Figure 6

Effect of 1,10-Phenanthroline on the Binding of DNA to Poly(ADP-ribose) Polymerase. Poly(ADP-ribose) polymerase (10 pmol/ml) was incubated in reaction mix (pH 6.0, -NAD⁺, -DNA) for 20 min at 25°C with 1,10-phenanthroline. [³H]-ColE1 DNA (10 μg) was added, incubation was continued for 1 min and bound DNA (o) was measured by filter binding assay. Identically treated samples were measured for activity (●) after the addition of [³H]-NAD⁺ (0.1 mM) and DNA (10 μg) in place of [³H]-ColE1 DNA.



3. Interaction of Poly(ADP-ribose) Polymerase and DNA

Poly(ADP-ribose) polymerase has been shown to have an almost absolute requirement for DNA (Yoshihara, 1972; Niedergang et al, 1979), but the underlying basis for the DNA-enzyme relationship is not understood. Since poly(ADP-ribose) polymerase itself is a major acceptor of poly(ADP-ribose) (Yoshihara et al, 1977; Caplan et al, 1979; Ikai & Ueda, 1980; Kawaichi et al, 1981b) we have used a model system of DNA and purified polymerase to study the effect of poly(ADP-ribosyl)ation on DNA binding. In this section we report some of the parameters involved in the regulation of poly(ADP-ribose) polymerase activity which involves a tripartite relationship of polymerase, DNA and poly(ADP-ribose) glycohydrolase.

3a. Automodification of Poly(ADP-ribose) Polymerase: It had been noted previously that the inhibition of polymerase after a prolonged incubation was not due to the exhaustion of the substrate or to enzyme inactivation (Tanaka et al, 1979). Here we note the decline in enzyme activity with time after an incubation in the time course buffer with unlabelled NAD^+ at 25° (Table VIII). No inhibition occurred if DNA was also omitted during the pre-incubation (data not shown), suggesting the need for enzyme activity. Concurrent studies indicated that both chain length and chain number increased while the overall enzyme activity was decreasing (Table IX).

Table VIII

The Effect of Pre-Incubation with NAD^+ on Poly(ADP-ribose) Polymerase Activity. Poly(ADP-ribose) polymerase (30 pmol/ml) was incubated in reaction mix containing 35 μM NAD^+ . Aliquots of 0.1 ml were removed at various times and assayed for enzyme activity by incubating with [^3H]- NAD^+ for 2.5 min.

PRE-INCUBATION TIME

ACTIVITY

min	pmol/min/ μ g protein	%
0	76.2	100
1	66.5	87
2	54.4	71
5	24.1	32
10	20.6	27
20	11.5	15
30	10.3	13
45	5.9	8
60	6.5	9
90	2.9	4

Table IX

Analysis of Poly(ADP-ribose) Formation with Time.
Poly(ADP-ribose) polymerase (5.4 μ mol/ml) was
incubated in reaction mixture for various times,
precipitated with 20% trichloroacetic acid and
analyzed for poly(ADP-ribose) formation and chain
length.

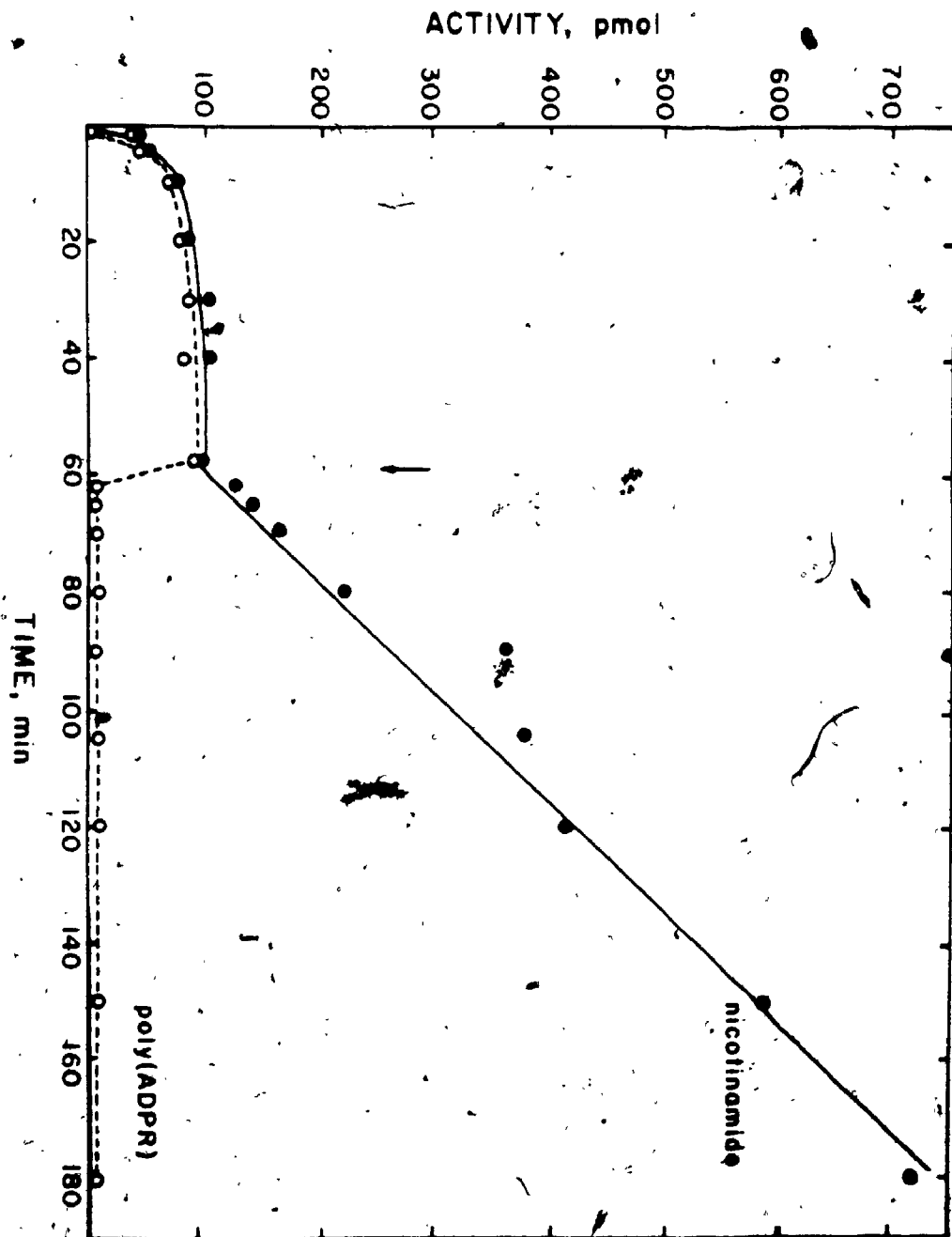
TIME	ADP-RIBOSE INCORPORATION	CHAIN LENGTH	CHAIN NUMBER
min	mol/mol enzyme		polymers/enzyme
0.5	16	6.9	2.4
2	56	15.9	3.5
10	121	21.6	5.6
60	221	19.3	11.5

A double-label assay monitoring nicotinamide release and poly(ADP-ribose) formation was used to study the role of poly(ADP-ribose) glycohydrolase in regulating poly(ADP-ribose) polymerase activity. Fig. 7 illustrates the increasing inhibition of polymerase activity on prolonged incubation and indicates the equivalence of the nicotinamide release and poly(ADP-ribose) incorporation assays. The subsequent addition of glycohydrolase degraded the poly(ADP-ribose), reversed the inhibition and resulted in polymerase activity with linear kinetics as determined by nicotinamide release. The addition of heat-inactivated glycohydrolase (heated at 60°C for 5 min) failed to reactivate the polymerase, indicating the requirement of a catalytic function (data not shown). However, since the glycohydrolase preparation was not completely purified, some other catalytic function is not ruled out. Other experiments, not presented here, have shown that after glycohydrolase treatment the average chain length of poly(ADP-ribose) was reduced from 18.0 to 1.4 residues. These experiments indicate that the polymerase was turned off by self-modification and turned on by coupling with glycohydrolase.

3b. Role of DNA Binding: Does auto-modification inhibit polymerase activity because of saturation of the poly(ADP-ribose) acceptor sites or because of an altered affinity for DNA? The former possibility seemed unlikely because

Figure 7

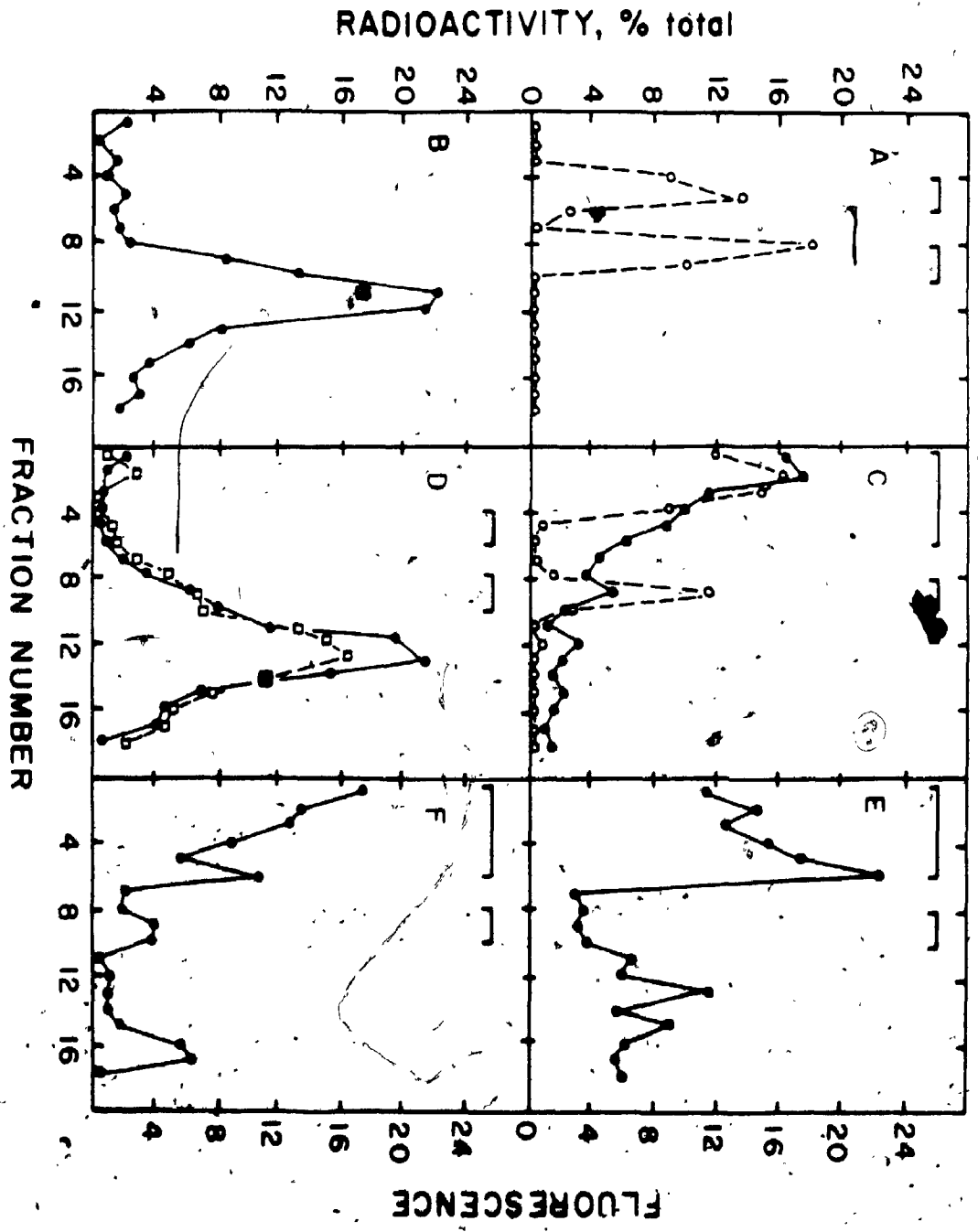
Coupling of Poly(ADP-ribose) Polymerase with Poly(ADP-ribose) Glycohydrolase. The double-label assay was used to monitor polymerase activity by following ADP-ribose incorporated (o) and nicotinamide released (●). The reaction vessel contained 11 pmol polymerase/ml. Glycohydrolase (0.13 mg/ml) was added as indicated (↓).



the level of inhibition seemed to depend, for example, on the particular DNA that was used (see below). To test the latter possibility we studied polymerase-DNA interactions by sucrose gradient centrifugation using ColE1 plasmid DNA. With ColE1 DNA, the total incorporation was reduced by 80% compared with similar experiments with calf thymus DNA (Fig. 7), but otherwise there was a similar inhibition of polymerase. Sucrose gradient centrifugation analysis indicated that after a 60 min incubation in the absence of NAD^+ , polymerase and DNA co-sedimented (Fig. 8C) at a position further down the gradient than DNA or polymerase alone (Fig. 8A,B). Parenthetically, we note that the polymerase appears to bind preferentially to form I DNA rather than form II under these conditions. After incubation of the enzyme with NAD^+ for 60 min, not only was the enzyme inhibited, it was also dissociated from DNA (Fig. 8D). Both enzyme activity and labelled poly(ADP-ribose) sedimented in the upper part of the gradient. A 15 min incubation following the addition of glycohydrolase to the inhibited polymerase restored the DNA-polymerase complex (Fig. 8E). The use of snake venom phosphodiesterase in place of glycohydrolase to cleave poly(ADP-ribose) also resulted in a similar reassociation of polymerase and DNA (Fig. 8F). This supports our contention that glycohydrolase functions in reactivating the polymerase by removing poly(ADP-ribose) residues. In the phosphodiesterase experiment, the normal acceptor sites for poly(ADP-ribose)

Figure 8

Influence of Poly(ADP-ribosylation) on the Binding of DNA to Poly(ADP-ribose) Polymerase. Interaction of polymerase (48 pmol) and ColE1 DNA (48 pmol, 50% form I/50% form II) was observed by sucrose gradient sedimentation. Samples were prepared in reaction mix containing (A) ColE1 DNA, (B) polymerase, (C) ColE1 DNA and polymerase, (D) ColE1 DNA, polymerase, [³H]-NAD⁺ incubated for 60 min, (E,F) 60 min incubation of ColE1 DNA, polymerase, [³H]-NAD⁺ followed by a 15 min incubation with 0.4 mg glycohydrolase (E) or 1.0 unit snake venom phosphodiesterase (F). DNA (o) was measured by fluorescence assay, polymerase (●) by the standard assay (NAD⁺ at 10 μM) and [³H]-poly(ADP-ribose) (□) by precipitation in 20% trichloroacetic acid. Sedimentation is from right to left. DNA is also indicated by the brackets.

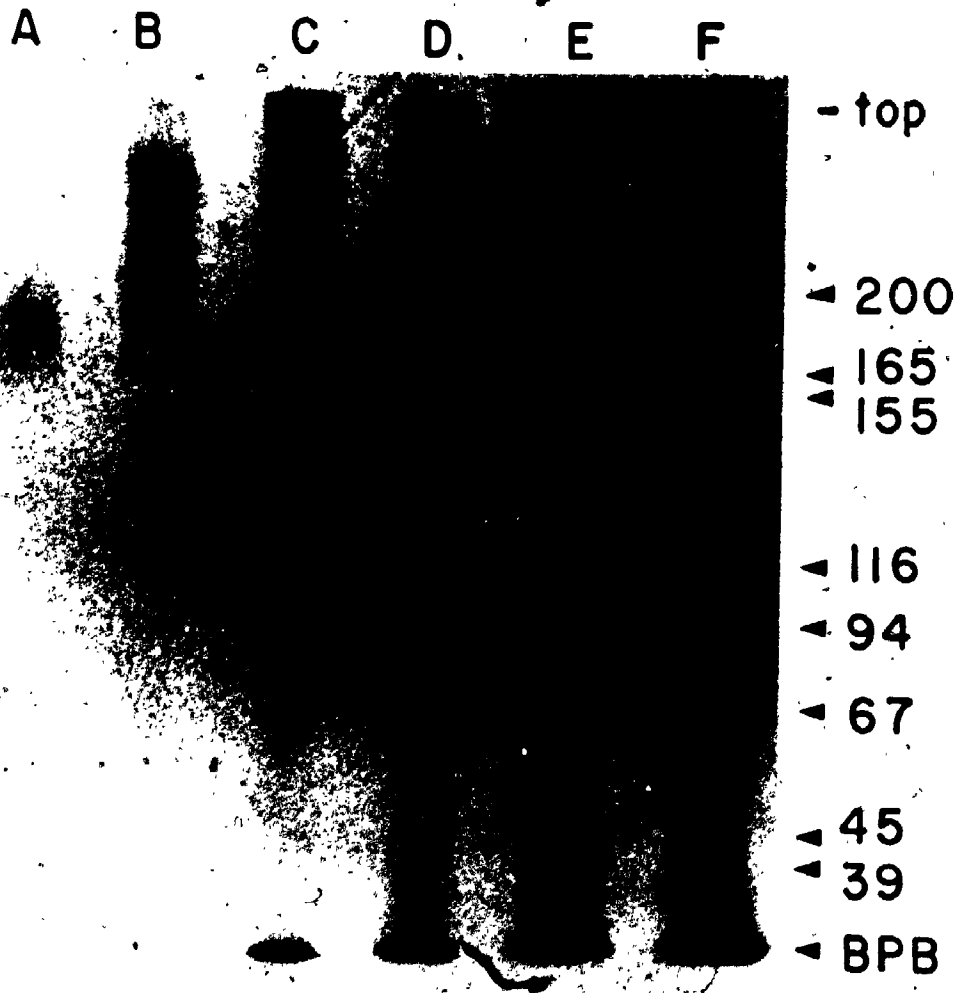


on the polymerase were destroyed by phosphodiesterase treatment; however, the presence of the enzyme was still detectable. We noted that both the histone in the assay mixture as well as some of the enzyme were being modified (results not shown). These experiments show that all of the poly(ADP-ribose) co-sediments with the enzyme (Fig. 8D).

In order to distinguish between free poly(ADP-ribose) absorbed to the enzyme and poly(ADP-ribose) covalently bound to the enzyme, the products of the reaction mixture were analyzed by SDS/polyacrylamide gel electrophoresis. As shown in Fig. 9, the bulk of the radioactivity migrated in the upper portion of the gel, suggesting a molecular weight considerably greater than that of the free enzyme. The treatment of the reaction mixture with proteinase K or 0.1 M NaOH resulted in the release of poly(ADP-ribose) and the migration of the labelled product at the gel front (Fig. 9E, F). These results indicated that the bulk (92-96% by densitometry analysis) of the poly(ADP-ribose) was covalently bound to the enzyme. Furthermore, the addition of free poly(ADP-ribose) in concentrations up to 5 μ M did not inhibit the enzyme (data not shown). Similar results have been reported for the rat liver enzyme (Kawaichi et al, 1981b). In summary, the experiments indicate that auto-poly(ADP-ribosyl)ation inhibited the polymerase activity by altering the DNA-enzyme complex.

Figure 9

Electrophoretic Analysis of Poly(ADP-ribose) Polymerase Automodification. Samples were incubated in reaction mix ($[^3\text{H}]\text{-NAD}^+$ at $58 \mu\text{M}$) for 0.5 min (A), 2 min (B), 10 min (C), 60 min (D) and precipitated with 20% trichloroacetic acid. Separate samples were treated after the 60 min incubation with proteinase K (E) or 0.1 M NaOH (F) prior to termination of the reaction. The pellets were rinsed twice with cold acetone, suspended in neutral gel sample buffer and subjected to neutral-SDS/polyacrylamide gel electrophoresis (7.5% polyacrylamide). The gel was analyzed by autoradiography. Protein standards, visualized by Coomassie blue staining, were run concurrently and included myosin, RNA polymerase, β -galactosidase, phosphorylase b, bovine serum albumin and ovalbumin. Unmodified polymerase migrates at Mr 114000. BPB = bromphenol blue.



Mr. 10⁻³

3c. Influence of Mg^{2+} and Histones: Mg^{2+} , polyamines and histones are known to increase the activity of poly(ADP-ribose) polymerase (Okayama et al, 1977; Tanaka et al, 1979; Yamada & Sugimura, 1973; Bryne et al, 1978; Tanigawa et al, 1980). Fig. 10 shows that increasing Mg^{2+} concentrations resulted in an elevated enzyme activity. Similar activation has been noted with Mn^{2+} and Ca^{2+} (Niedergang et al, 1979). Sucrose gradient centrifugation analysis indicated that after incubation of polymerase without NAD^+ in 0.1 mM or 2 mM $MgCl_2$ at $0^\circ C$ typical ColE1 DNA-enzyme complexes were observed (Fig. 11A,B). However, if these reaction mixtures were incubated at $25^\circ C$ for 60 min. with NAD^+ , then the enzyme was dissociated from DNA in the 0.1 mM $MgCl_2$ reaction mixture (Fig. 11C) while in the 2 mM $MgCl_2$ sample, a considerable quantity of enzyme remained associated with DNA (Fig. 11D). Control experiments, not shown here, indicated that the Mg^{2+} concentrations used in the gradients did not influence the results. These results indicated that the stimulation of enzyme activity by Mg^{2+} was correlated with an increased affinity of enzyme and DNA.

It has previously been suggested that Mg^{2+} stimulates polymerase activity by activating contaminating nucleases (Benjamin & Gill, 1980b). We have checked this possibility for our experimental conditions by incubating both calf thymus and ColE1 DNA with varying amounts of pancreatic DNase I. We noted a stimulation of polymerase activity by

Figure 10

The Effect of $MgCl_2$ Concentration on Poly(ADP-ribose) Polymerase Activity. Polymerase (5 pmol/ml) was incubated in reaction mixture containing either 0 mM $MgCl_2$ (\bullet), 0.1 mM $MgCl_2$ (\circ), or 2 mM $MgCl_2$ (\square). Aliquots of 0.1 ml were removed, precipitated with 20% trichloroacetic acid and analyzed for poly(ADP-ribose) formation.

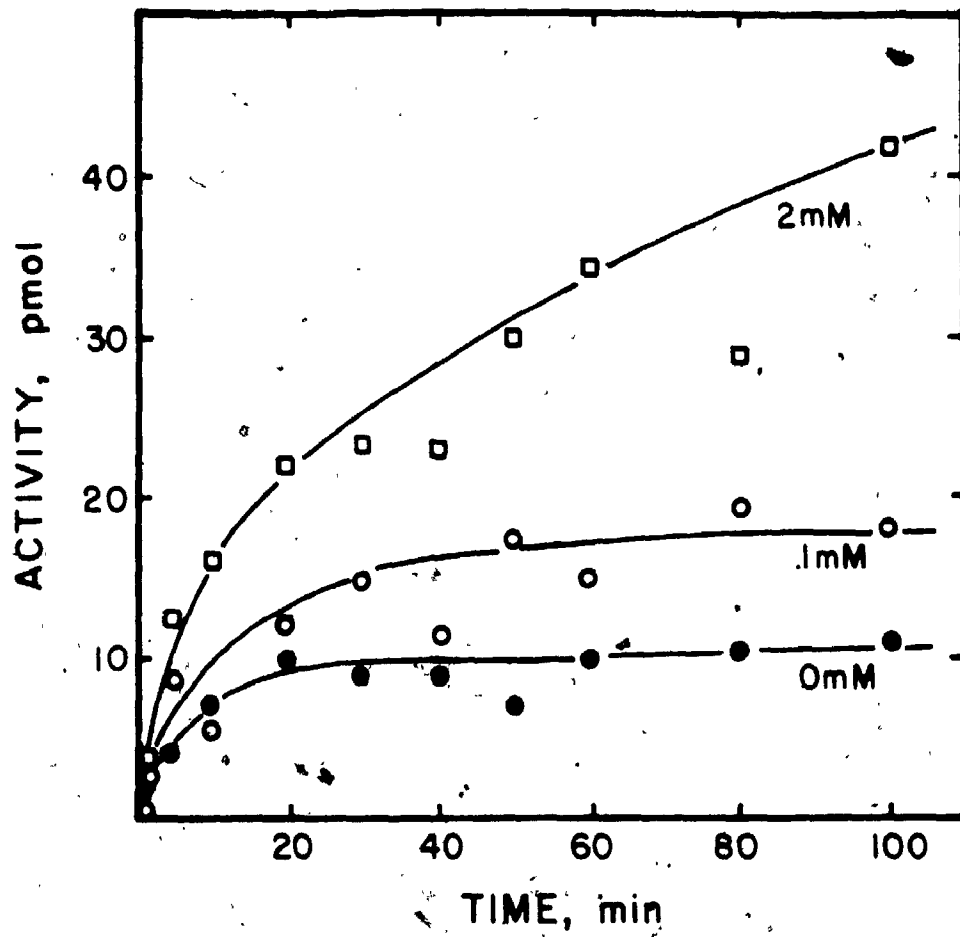
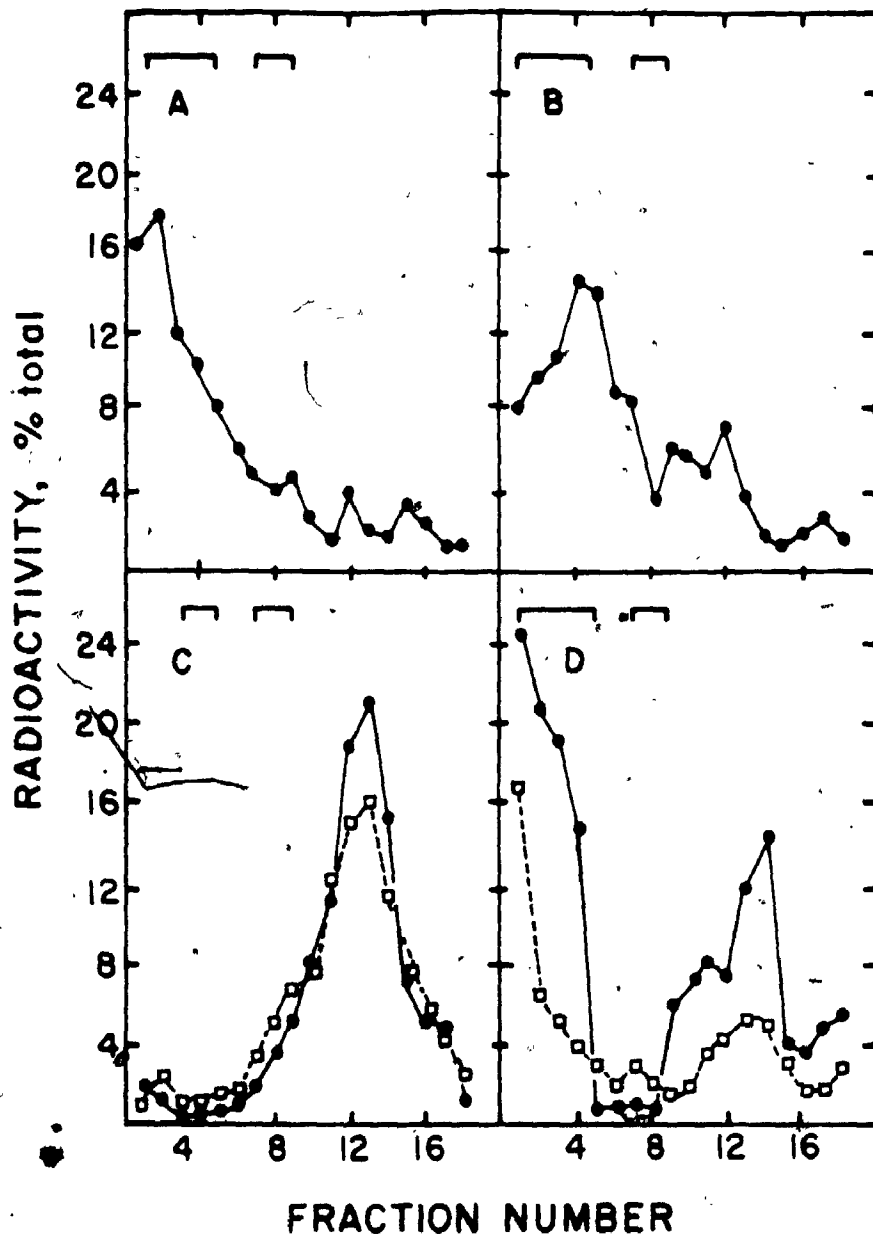


Figure 11

Effect of $MgCl_2$ on the Interaction of Poly(ADP-ribose) Polymerase and DNA. Samples of polymerase (48 pmol/ml) and Cole1 DNA (48 pmol/ml; 50% form I/50% form II) were prepared by a 60 min incubation in reaction mix containing 0.1 mM $MgCl_2$ (A), 2 mM $MgCl_2$ (B), 0.1 mM $MgCl_2$ and [3H]- NAD^+ (C) and 2 mM $MgCl_2$ and [3H]- NAD^+ (D). The DNA-polymerase interaction was observed by sucrose gradient sedimentation. Gradients contained $MgCl_2$ equivalent to the applied samples. DNA, measured fluorometrically, is indicated by brackets, polymerase (\bullet) was assayed in the standard assay (NAD^+ at 58 μM) and [3H]-poly(ADP-ribose) (\square) determined by precipitation with 20% trichloroacetic acid. Sedimentation is from right to left.



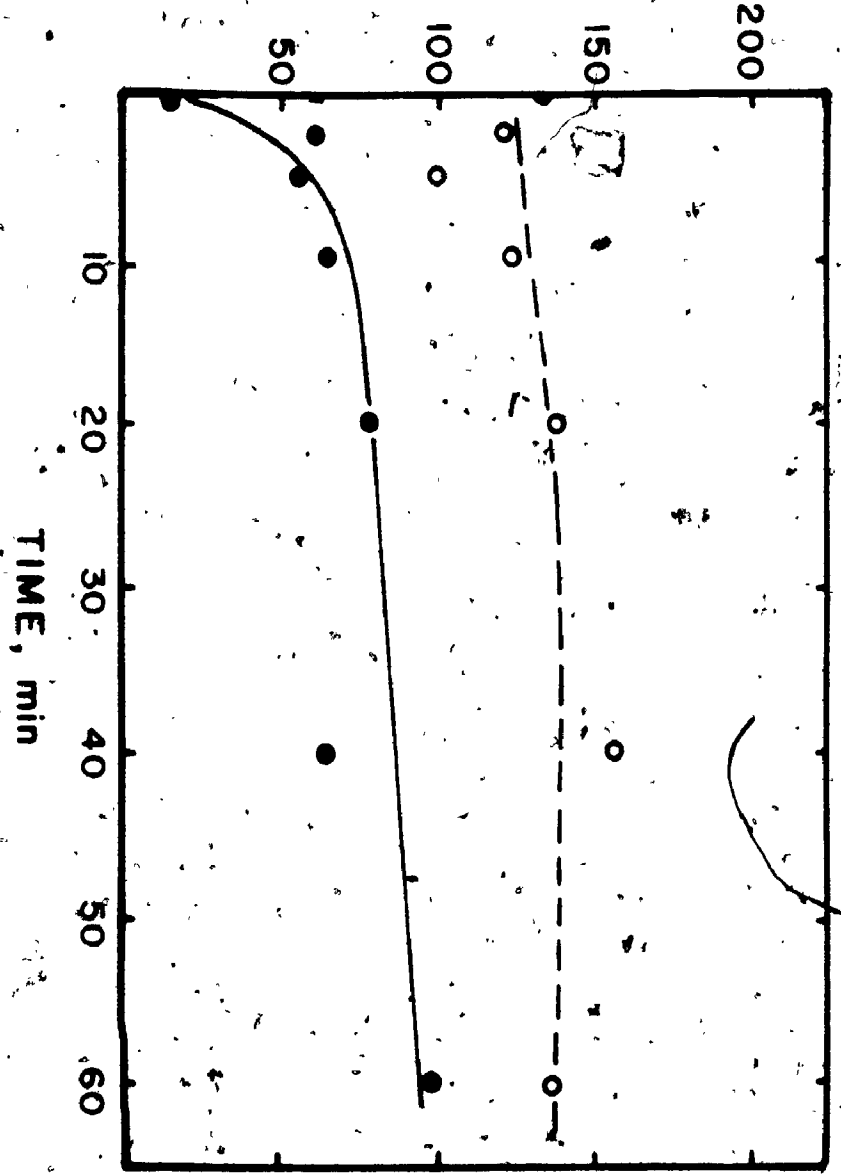
the action of DNase on ColE1 DNA, but there was no significant increase in activity with the already highly nicked calf thymus DNA (Fig. 12). Similar results were obtained with calf thymus DNA incubated with 0.08 and 0.6 $\mu\text{g}/\text{ml}$ DNase (results not shown). These results suggest (but do not prove) that the stimulation by Mg^{2+} observed for calf thymus DNA (Fig. 10) was not due to stimulation of a nuclease.

Histones not only serve as acceptors of poly(ADP-ribose) (Bryne et al, 1978; Kawaichi et al, 1981) but also stimulate the polymerase reaction (Okayama et al, 1977; Tanaka et al, 1979). In the absence of histones the poly(ADP-ribosyl)ated enzyme was inhibited and sedimented in a sucrose gradient at the position of the uncomplexed enzyme after incubation with NAD^+ (Fig. 13B). Fig. 13A indicates the position of the enzyme prior to incubation with NAD^+ . The addition of histone H1 to a modified and inhibited polymerase led to an immediate increase in enzyme activity (inset, Fig. 13) and sucrose gradient centrifugation analysis indicated that most of the enzyme and some of the labelled poly(ADP-ribose) sedimented with the DNA (Fig. 13C). However the rest of the enzyme appeared somewhat heterogeneous and was largely separated from the labelled poly(ADP-ribose). A possible explanation for the separation of the enzyme from poly(ADP-ribose) is that only a fraction of the enzyme molecules were modified and that the poly(ADP-ribose) kept the active enzyme in a complex or

Figure 12

Activity of Poly(ADP-ribose) Polymerase with DNase I Treated DNA. Activity of the enzyme was measured with both calf thymus DNA (o) and ColE1 (●) plasmid DNA (96% form I/4% form II) after treatment with 0.38 μ g/ml DNase I for various lengths of time.

ADP-RIBOSE INCORPORATED, pmol



22
OF / DE 22

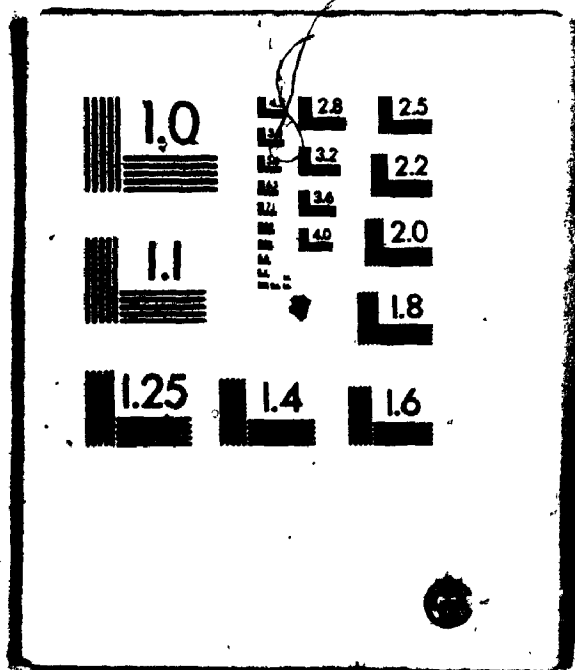
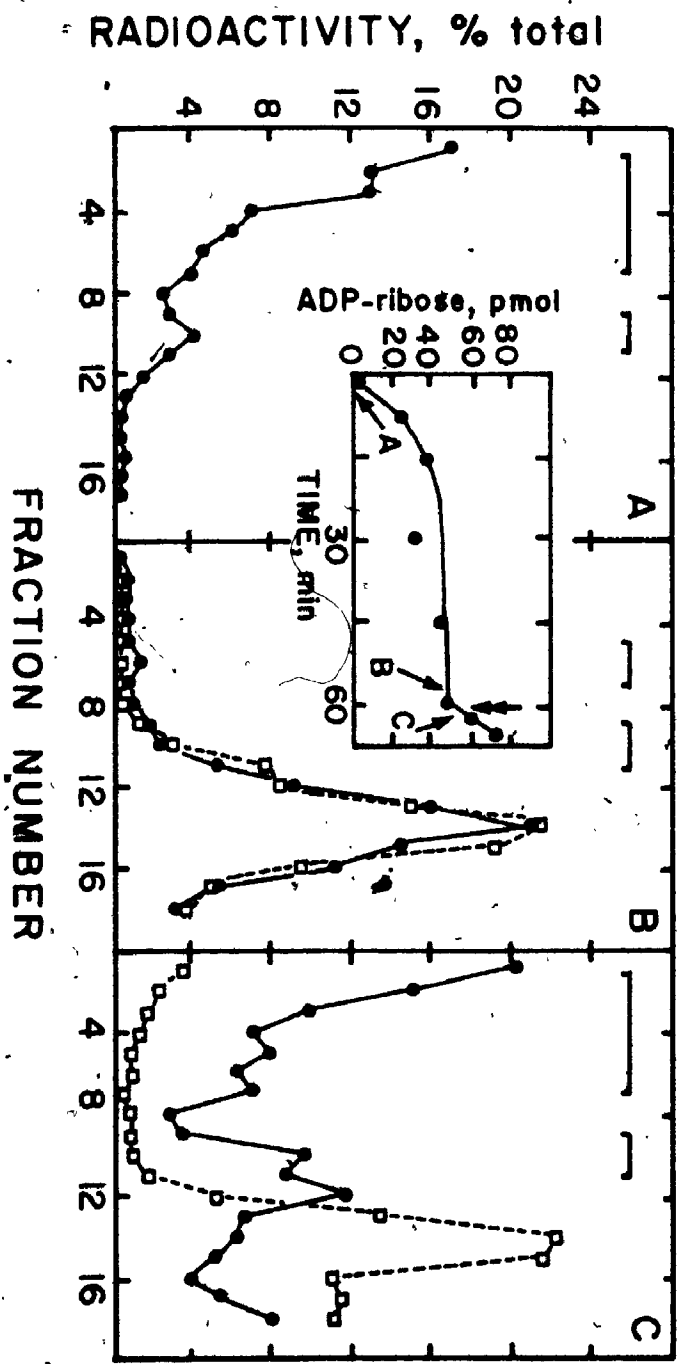


Figure 13

The Effect of Histone H1 on Poly(ADP-ribose) Polymerase Activity and Interaction with DNA. The inset shows the time course of poly(ADP-ribose) formation in reaction mix in the absence of histones and following the addition of 0.1 mg/ml histone H1 at 60 min (\downarrow). As well, the times at which samples were removed for sucrose gradient analysis are indicated (A,B,C). Samples containing Cole1 DNA were identically prepared to those indicated in the inset (A,B,C) and DNA-protein interactions analyzed by sucrose gradient sedimentation. (A) Sedimentation of enzyme (80 pmol/ml) and Cole1 DNA (1.8 mg; 50% form I/50% form II) at zero time; (B) sedimentation after incubation with NAD^+ for 60 min; (C) analysis made immediately after addition of 0.1 mg/ml histone H1. Panels indicate polymerase activity (\bullet) measured by the standard assay (NAD^+ at 58 μM) and [^3H]-poly(ADP-ribose) (\square) determined by 20% trichloroacetic acid precipitation. DNA, measured by fluorescence assay, is indicated by brackets. Sedimentation is from right to left.



aggregated form with the modified enzyme. The addition of histones dispersed this complex and the unmodified enzyme formed a different type of aggregate. The sedimentation behavior of the enzyme under these conditions indicated an aggregated form, confirming a previous report of a high molecular-weight form of the enzyme (Kristensen & Holtlund, 1978). Similar results were obtained when total histone preparations replaced histone H1 (results not shown).

DISCUSSION

1. Poly(ADP-ribose) Polymerase Purification

The purification procedure for calf thymus poly(ADP-ribose) polymerase described here is basically similar to several other published procedures (Yoshihara et al, 1978; Ito et al, 1979). We have modified the procedure by using Matrex gel red A as a high affinity chromatographic medium and eliminated the gel filtration step. These modifications resulted in a more rapid purification and an enzyme preparation of comparable purity with an improved yield (Fig.2, Table I).

2. Poly(ADP-ribose) Polymerase, a Zinc Metalloenzyme

2a. Identification of Zinc Content: Previous studies indicated that 1,10-phenanthroline did not inhibit at pH 8.0 (Carter & Berger, 1982), however at pH 6.0, 1,10-phenanthroline clearly inhibits (Table II). This pH-dependent inhibition may be related to the acid-base properties of the ligands coordinating the metal (Vallee & Wacker, 1970) and to the availability of the metal to the solvent as demonstrated by the ^{65}Zn exchange (Table VI). In particular; the pH range for inhibition suggests the involvement of histidine, which is a favored ligand for zinc, or a zinc-coordinated water molecule such as that observed in carbonic anhydrase (Cheblowski & Coleman, 1976)

and in the catalytic site of alcohol dehydrogenase (Sytkowski & Vallee, 1976). The inhibition by 1,10-phenanthroline was time-dependent and not reversible by dilution or the addition of Co^{2+} or Zn^{2+} (data not shown) suggesting that this chelator functions by removal of the metal from the enzyme (Vallee & Wacker, 1970). The poor inhibition by neocuprone (2,9-dimethyl-1,10-phenanthroline) as compared to 1,10-phenanthroline or 2,2'-bipyridine suggests that the metal may be sterically available to a narrow range of compounds.

The inhibition by chelators as well as the atomic absorption spectroscopy studies reported here indicate that poly(ADP-ribose) polymerase is a zinc metalloenzyme. This conclusion is further strengthened by results of the ^{65}Zn exchange. Metal analyses indicated approximately one g-atom of zinc per mole of enzyme (Table V). Furthermore the inhibition of the enzyme by 1,10-phenanthroline suggests chelation of zinc in the enzyme. However, the inhibition of poly(ADP-ribose) polymerase by 1,10-phenanthroline might result from the degradation of DNA, an essential component in the reaction. A 1,10-phenanthroline-cuprous complex together with hydrogen peroxide and a thiol has been shown to cause cleavage of DNA (Sigman et al, 1979). This latter possibility seemed unlikely to account for the inhibition for the following reasons: (1) the inhibition of poly(ADP-ribose) polymerase by 1,10-phenanthroline also occurred in the presence of catalase which

effectively prevented DNA degradation (Table IV; Marshall et al, 1981). (ii) poly(ADP-ribose) polymerase was inhibited by 2,2'-bipyridine, an analogue of 1,10-phenanthroline which does not cleave DNA (Coleman, 1983) (iii) the inhibition of poly(ADP-ribose) polymerase by 1,10-phenanthroline was pH-dependent while the degradation of DNA was pH-independent (data not shown).

Although these studies indicate the presence of zinc in poly(ADP-ribose) polymerase, they do not necessarily demonstrate that zinc is required for enzyme activity. In this context it should be noted that similar observations suggested an involvement of zinc in DNA polymerase I activity but subsequent experiments indicated that a zinc-deficient enzyme was enzymatically active (Walton et al, 1982; Ferrin et al, 1983). In order to show a more direct relationship of zinc content and poly(ADP-ribose) polymerase activity, the enzyme was dialyzed against buffers containing 1,10-phenanthroline under inhibiting (pH 6.0) and non-inhibiting (pH 8.0) conditions. These studies indicated a parallel relationship of zinc content and enzyme activity (Table VII), thus supporting the contention that zinc is involved in the enzyme activity.

Some of the possible errors involved in the analysis of the zinc content of the enzyme should be noted. The variations in the zinc analysis are indicated in Table VII. The protein analysis in Table V might be subject to some error since the quantity of protein measured was at

the lower limits (<5 μg) of the assay. This is not the case for Table VII where larger amounts (10-20 μg) of protein were analyzed. Another possible source of error might involve the loss of zinc during the dialysis procedure. Although it could be argued that zinc was non-specifically adsorbed to the enzyme, the failure of Cd^{2+} to compete with ^{65}Zn in the exchange assay would argue for a specific binding of zinc in the enzyme.

2b. Zinc Requirement in the Enzyme Mechanism: Zinc has been identified as a constituent of most classes of nucleotidyl transferases (Vallee & Falchuk, 1981; Coleman, 1983). The DNA-dependent metalloenzymes which have been studied in greatest detail, such as the DNA-dependent RNA polymerases, may require zinc for the interaction of DNA with the protein (Coleman, 1983; Mildvan & Loeb, 1979). Our kinetic studies suggest that zinc may have a similar role in poly(ADP-ribose) polymerase and may indicate a catalytic function. The DNA-binding results indicate poly(ADP-ribose) polymerase has a general DNA binding domain as well as a more specific site involving a zinc-DNA interaction. A general DNA-binding domain for the enzyme is expected because of the basic properties ($\text{pI}=9.8$) of the enzyme (Ito et al, 1979).

What are the implications of these findings? While zinc is a well-known component of many enzymes, the observation that zinc-depleted Euglena gracilis cells were

blocked in the G₂ phase of the cell cycle suggests a more specific and critical zinc requirement (Falchuk et al, 1975). Studies of poly(ADP-ribose) polymerase HeLa cell mutants have shown a requirement for poly(ADP-ribose) polymerase activity in the G₂ phase of the cell cycle (to be published) suggesting a possible connection between zinc deficiency, poly(ADP-ribose) polymerase and G₂ phase reactions. The effect of zinc-deficiency on the chromatin arrangement of Euglena cells (Stankiewicz et al, 1983) might also correlate with the role of poly(ADP-ribosyl)-ation in the maintenance of chromatin structure (Wong et al, 1982; Poirier et al, 1982). From a mechanistic point of view, the finding that zinc is involved in a catalytic role and/or in the interaction of DNA and enzyme introduces a new and possibly significant clue for understanding the puzzling requirement of DNA for poly(ADP-ribose) polymerase activity. The known requirement of nicks or double-stranded breaks in DNA for enzyme activity (Ohgushi et al, 1980; Benjamin & Gill; 1980b) suggests the possibility that zinc might be involved in the interaction with the exposed nucleotide. If such interactions occurred with poly(ADP-ribose) polymerase, they could explain in part the requirement of nicks or breaks in DNA for enzyme activity.

3. Interaction of Poly(ADP-ribose) Polymerase and DNA

3a. Automodification and DNA Binding: It has previously

been observed that poly(ADP-ribose) polymerase required DNA for activity (Yoshihara, 1972; Niedergang et al, 1979) and recently Yoshihara et al (1981) have reported that the poly(ADP-ribosyl)ated polymerase has a decreased affinity for DNA. Our studies have been concerned with the mechanism by which polymerase is regulated. We conclude that the self-modification of the polymerase (Fig. 9) is part of an auto-regulatory mechanism for turning off the enzyme (Table VIII, Fig. 7) and that this modulation results from a decreased affinity of the modified enzyme for DNA (Fig. 8). This modified and inhibited enzyme was reactivated by poly(ADP-ribose) glycohydrolase (Fig. 7) which removed poly(ADP-ribose) residues and restored the DNA-poly(ADP-ribose) polymerase complex (Fig. 8). The restoration of enzyme activity after glycohydrolase treatment indicated that poly(ADP-ribose), as compared to single ADP-ribose residues, has a critical role in enzyme regulation since glycohydrolase does not remove the ADP-ribose groups adjacent to the protein (Ueda et al, 1972).

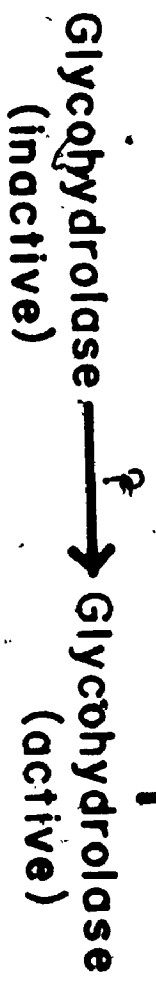
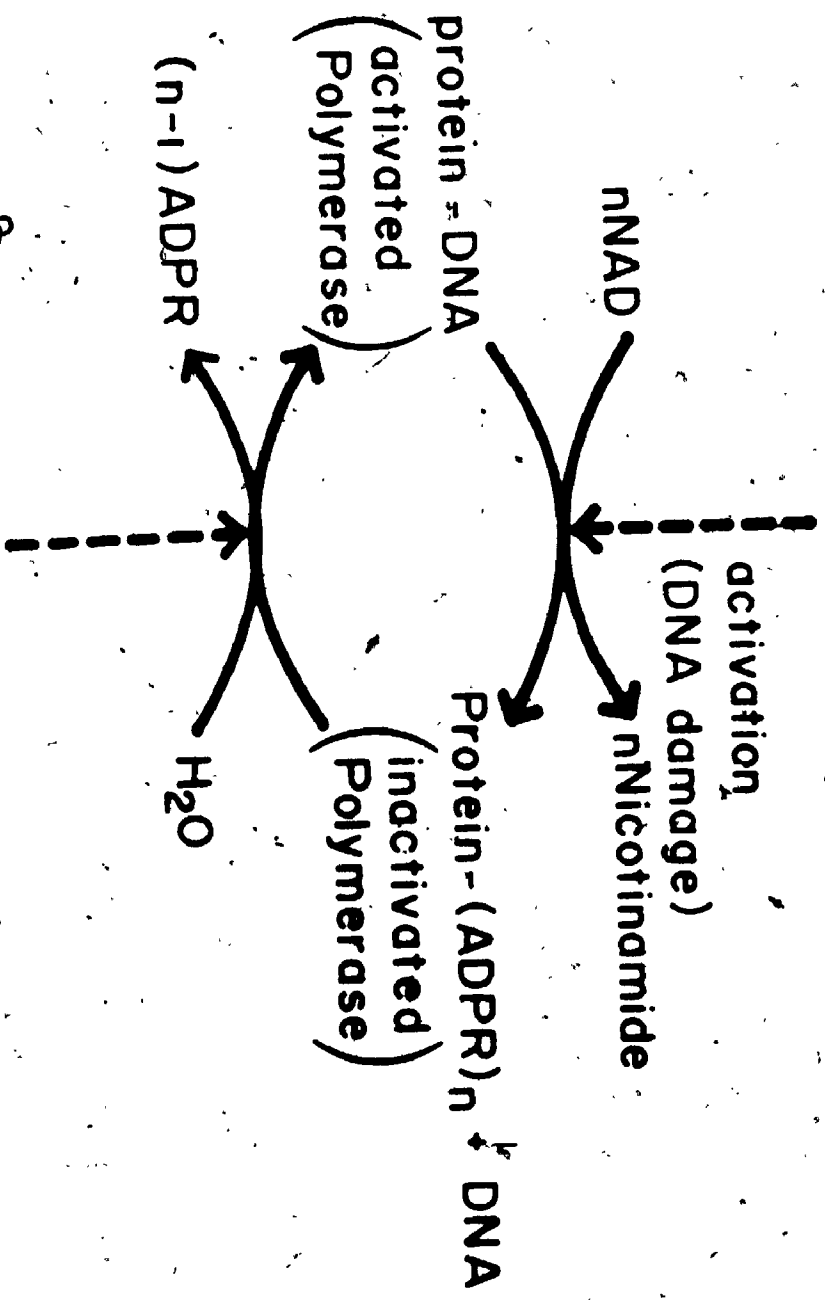
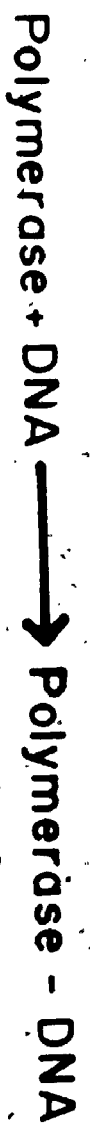
We have noted that Mg^{2+} and histones, which have previously been shown to stimulate polymerase activity (Tanaka et al, 1979), also appear to activate the enzyme by influencing the DNA-enzyme interactions. The addition of Mg^{2+} increased enzyme activity (Fig. 10) as well as the amount of enzyme remaining bound to DNA (Fig. 11). Similarly, the addition of histone H1 to a poly(ADP-

ribosyl)ated polymerase resulted in the reactivation of the enzyme (inset, Fig. 13) and the reappearance of the DNA-enzyme complex (Fig. 13). Although the transfer of poly(ADP-ribose) residues from the modified enzyme to histone is a possible mechanism of reactivation, this is probably not occurring since it has been shown that covalently bound poly(ADP-ribose) is not transferred to histone (Kawaichi et al, 1981b; Yoshihara et al, 1981). Furthermore, we noted that the addition of the polymerase inhibitor nicotinamide (5 mM) just prior to the addition of histone H1 did not prevent the formation of the enzyme-DNA complex, indicating that the transfer of poly(ADP-ribose) to histone was not involved (data not shown). This experiment also indicated that the poly(ADP-ribose) found at the bottom of the gradient was not newly synthesized. Possibly Mg^{2+} and histone increased the enzyme activity by neutralizing the negative charges on poly(ADP-ribose) or DNA (Kawamura et al, 1981) to promote increased stability of the DNA-enzyme complexes.

3b. A Shuttle Mechanism for Modulating DNA-Protein Interactions: Our experiments have indicated that by coupling poly(ADP-ribose) polymerase and glycohydrolase, we have a shuttle mechanism for moving proteins on and off DNA. In Fig. 14 we summarize our proposal for the shuttle and the mechanism involved in the control of poly(ADP-ribose)ation. As shown by several investigators, poly-

Figure 14

Proposed Mechanism for the Poly(ADP-ribose) Shuttle
and the Control of Poly(ADP-ribosylation).



(ADP-ribose) polymerase requires nicked DNA or DNA ends for activity (Benjamin & Gill, 1980b; Ohgushi et al, 1980) and DNA damage appears to activate the enzyme (Benjamin & Gill, 1978; Durkacz et al, 1980a). We presume that the DNA functions as an effector. The activated polymerase poly-(ADP-ribosyl)ates itself and, in time, the polymerase dissociates from the DNA. Although we have no experimental evidence, we would like to suggest that other acceptor proteins might also function in this shuttle reaction. Glycohydrolase completes the shuttle by restoring the polymerase-DNA complex. Clearly, complete control of poly-(ADP-ribosyl)ation would require the modulation of the glycohydrolase as well since an active, accessible glycohydrolase would abort any poly(ADP-ribosyl)ation. Our proposal for the control of poly(ADP-ribose) polymerase covers the situation in which other acceptor proteins are absent. If another acceptor protein (histone H1) were present (inset, Fig. 13), the acceptor protein appears to provide an override mechanism to allow the enzyme to function. It is not possible to extrapolate from these selective in vitro conditions to the affairs of the cell, or for that matter, to interactions involving other poly(ADP-ribosyl)ated proteins. Nevertheless, a need for a shuttle mechanism of some sort for modulating DNA-protein interactions seems to be almost self evident.

SUMMARY

The investigations described in this thesis have been performed in order to answer the fundamental question: what is the relationship between poly(ADP-ribose) formation and DNA? This question was approached from two viewpoints, one concerned with the mechanism by which DNA activates poly(ADP-ribose) polymerase and the second examining the effect of poly(ADP-ribosyl)ation on the activity and DNA-binding properties of modified proteins.

In order to undertake the biochemical studies involving the interaction of poly(ADP-ribose) polymerase and DNA, it was necessary to purify sufficient amounts of highly purified, DNA-dependent enzyme. The protocol which was finally used resembled several published procedures but the modifications made both shortened the preparation time and improved the final yield.

A number of conclusions were made concerning the relationship between DNA and poly(ADP-ribose) polymerase:

1. Poly(ADP-ribose) polymerase is a zinc metallo-protein containing one μ -atom zinc per mole protein.

2. Zinc is involved in the specific interaction between DNA and poly(ADP-ribose) polymerase which is required for activation of the polymerase.

3. Auto-modification of poly(ADP-ribose) polymerase

reduces the affinity of the enzyme for DNA which in turn causes inactivation of the enzyme.

4. Removal of poly(ADP-ribose) by poly(ADP-ribose) glycohydrolase both reactivated the enzyme and restored the DNA-polymerase complex.

5. Histones and divalent cations modulated the interaction of DNA and poly(ADP-ribose) polymerase by neutralizing the charges on poly(ADP-ribose) and thereby increased the enzyme activity.

The identification of poly(ADP-ribose) polymerase as a zinc metalloenzyme may help in understanding the relationship between the active site and the DNA as well as the catalytic process.

The relationship between auto-modification, activity and DNA binding led to the proposal of a shuttle mechanism for the regulation of poly(ADP-ribose) polymerase and DNA-protein interactions. Recently, electron microscopic studies have confirmed the main points of our model (DeMurcia et al, 1983). Other recent studies suggest that the inhibition of DNA topoisomerase I and Ca^{2+} , Mg^{2+} -endonuclease by poly(ADP-ribosyl)ation occur by dissociation of the enzymes from DNA (Jongstra-Bilen et al, 1983; Yoshihara et al, 1983). These findings suggest that the shuttle model may function for other enzymes and be a general mechanism by which poly(ADP-ribosyl)ation functions in the cell.

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