COMPLEX POLYMERS OF ADP-RIBOSE OCCUR IN VITRO AND IN VIVO

DISSERTATION

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By

Rafael Alvarez-Gonzalez, M.S.
Denton, Texas

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Poly(ADP-ribose) metabolism is a chromatin associated process that is rapidly accelerated in response to DNA damage. It appears to be required for cellular recovery from DNA damage and may also be involved in other chromatin associated processes such as cellular differentiation, gene expression and adaptation to general environmental stress. The regulation of chromatin functions by poly(ADP-ribose) metabolism can be accomplished by either the covalent modification of specific nuclear proteins or by non-covalent interactions between poly(ADP-ribose) and other molecules in chromatin. While limited information concerning the covalent functions of poly(ADP-ribose) in vivo has been published, it has not previously been possible to estimate the complexity of poly(ADP-ribose) in vivo. Studies of polymer complexity have not been heretofore possible because of the lack of suitable methodology to evaluate polymer complexity.

The work presented here included the development of a highly sensitive method to estimate the size and complexity of poly(ADP-ribose). This involved radiolabeling of the precursor pools, purification of polymers using a boronate resin, polymer fractionation according to size by molecular

sieve chromatography and analysis of polymer complexity by enzymatic digestion to nucleotides which were quantified by strong anion exchange chromatography. A modified version of the method was also found to be suitable to determine polymer complexity under conditions where radiolabeling is not possible. This approach should be useful to estimate polymer complexity in tissues.

The method was used to determine the average polymer size and branching frequency of poly(ADP-ribose) synthesized in intact cells under conditions of recovery from DNA damage. Complex polymers of ADP-ribose of about 200 residues in size containing multiple points of branching were detected in vitro and in intact cells. The studies strongly suggest that the regulation of chromatin functions by poly(ADP-ribosylation) is likely to involve non-covalent interactions between this negatively charged polymer and other chromatin components. In addition, the methodology developed should also prove useful for the localization of complex polymers of ADP-ribose in specific regions of chromatin.

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INTRODUCTION

In the last quarter century, research in biochemistry has made significant contributions to the advancement of a wide range of related disciplines including medicine, genetics, molecular biology, microbiology, developmental biology and biophysics among others. Biochemistry spans several different areas that are classified in different groups such as nucleic acids, proteins, vitamins, lipids, carbohydrates, etc.. The subject of the work presented in this dissertation is related to the biochemistry of the vitamin NIACIN. Vitamin is derived from the term "vital amine" and is defined as a substance required in the animal diet in very small quantities that is indispensable for the growth and maintenance of the organism. Vitamins are synthesized by plants and microorganisms and their dietary requirement in the animal results from the evolutionary loss of the respective biosynthetic ability. We currently use the term niacin to refer to two compounds, nicotinic acid and nicotinamide.

Niacin and Pellagra

Our knowledge of nicotinic acid dates from 1867, when Huber first reported its synthesis by the oxidation of nicotine and related compounds (71). In 1873, Weidel studied the crystal structure of the salts of nicotinic acid (176). However, it was not isolated from natural sources until the early 1900's when it was found in the antiberi-beri concentrate of rice (158).

The first important breakthrough that ultimately led to our understanding of the function of niacin and related biomolecules was made by Goldberger in 1915 (57). He demonstrated that pellagra was a dietary deficiency disease, curable by nutrients referred to as the pellagra preventive factor. This factor was later identified as niacin. Reports of pellagra date from 1735 when Casal described "mal de la rosa" in Spain. The term pellagra derives from the latin pelle agra which means "rough skin" and was first introduced by Frapolli in 1771 (108). This pathologic condition has been described as the disease of the four d's: dermatitis, dementia, diarrhea and death (See reference (155) for a detailed review). Since it can be cured by niacin intake, pellagra is now usually called the disease of the three d's because death rarely occurs. Of the pellagra symptoms indicated above, dermatitis appears first because of the high sensitivity to sunlight. If the niacin

deficiency is mild, sunburn appears first followed by thickening, scaling, hyperkeratinization and pigmentation of the skin. Areas of the body exposed to sunlight are primarily affected. In the more advanced, chronic stages, skin lesions develop in moist contact areas, such as axillae, groin and the area under the breast.

Some of the etiological questions about this disease were answered by the study of the animal model of pellagra, black tongue in dogs (31). (See reference 49 for a detailed review). An important observation was made by Elvehjem and co-workers in 1937 when they demonstrated that niacin cured black tongue in dogs (44), and almost immediatly thereafter the same group of investigators demonstrated the curative effect of niacin on pellagra patients (99). In 1943, Handles encountered difficulty in the induction of black tongue in dogs when using a diet deficient in nicotinic acid (59), and the possible involvement of a second nutrient factor was suggested. About the same time in 1945, Krelh et al. showed that the amino acid tryptophan had a curative effect on pellagra and a possible metabolic relationship between tryptophan and niacin was suggested (101). The connection between tryptophan and a pellagra-like syndrome was clinically shown in 1956 in the genetic disorder known as Hartnup's disease (12). These patients manifest the typical clinical symptoms of pellagra. The problem however, is the lack of absorption and/or increased renal excretion

of tryptophan. Thus, in 1979 Milner showed that tryptophan was an essential nutrient for dogs (115), and the observations made by Krelh et al. in the early 1940's, when studying black tongue in dogs could be explained.

Niacin and the Anabolism of Nicotinamide Adenine Dinucleotide

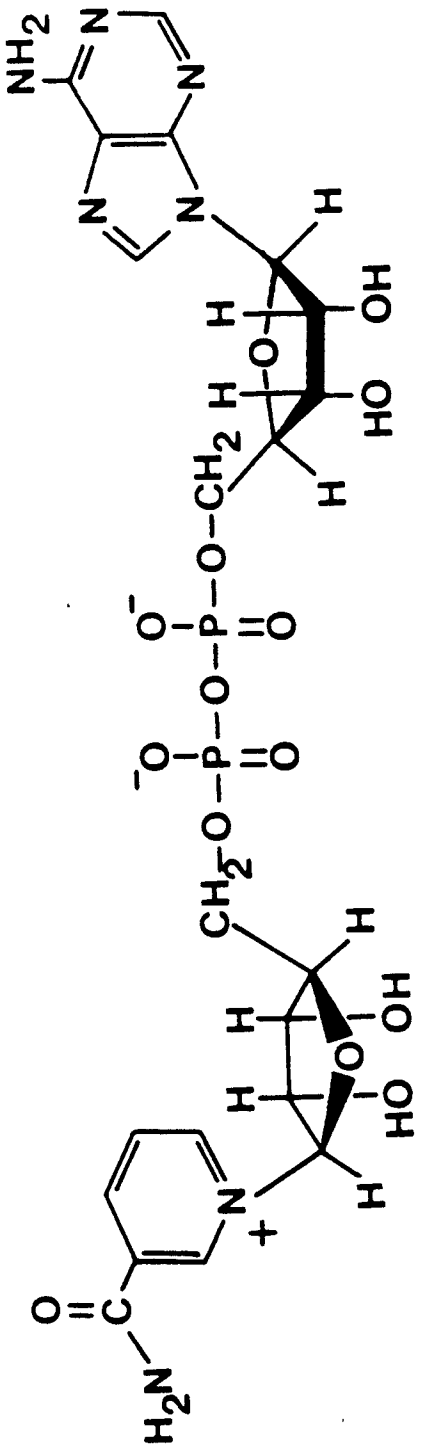
In the last three decades, it has been demonstrated that nicotinamide is essential for the activity of many enzymes as a component of the pyridine nucleotide coenzymes. The history of the nicotinamide containing coenzymes dates back to the work of Harden and Young in the early 1900's. They noted that the fermentation by yeast extract required both a non-dialyzable heat labile fraction and a dialyzable, heat stable component (62). Later, it was found that the dialyzable fraction contained two components or "coenzymes". Meyerhof and Lohman identified one of these components as adenosine triphosphate, which in addition is now known to be an energy reservoir. Von Euler and co-workers studied the other factor which they designated "cozymase" or coenzyme I and they reported the presence of adenosine 5'-monophosphate (AMP) in this compound. Soon after that, Warburg discovered that red blood cells contained a thermostable, dialyzable factor, required for the oxidation of glucose 6-phosphate to 6-phosphogluconate. They demonstrated that this factor was

composed of one molecule of adenine (Ade), two pentose units and three moles of phosphate per mole of coenzyme as well as one molecule of nicotinamide and thus the structure of coenzyme II was elucidated (175). In 1936, Von Euler and co-workers demonstrated that nicotinamide was also a component of coenzyme I (172). Finally, one year later they demonstrated that coenzyme I was composed of one mole of Ade one mole of nicotinamide, two moles of phosphoric acid, and two moles of d-ribose per mole of coenzyme I and the actual structure was proposed (173). The nomenclature of the pyridine nucleotide coenzymes was changed from coenzymes I and II to diphosphopyridine nucleotide or DPN and TPN or tryphosphopyridine nucleotide, once the chemical structure had been determined. Later, the enzyme commission (EC) of the International Union of Biochemistry proposed the names of nicotinamide adenine dinucleotide or NAD^+ and $NADP^+$ or nicotinamide adenine dinucleotide phosphate, which are now the most widely used terms. The reduced forms of these coenzymes are referred to as NADH and NADPH. The oxidized forms of the pyridine nucleotides are more stable than the reduced forms under acidic conditions. The reduced forms undergo a very rapid anomerization from the β-configuration to a mixture of the α and β forms in acidic solutions (78,140). However, under alkaline conditions, the reduced forms are more stable than the oxidized forms, since the N-glycosylic linkage of the oxidized form between the ribose

and the nicotinamide ring is cleaved to generate ADP-ribose and nicotinamide. It is important to note that only the β - configuration of NAD^+ appears to be active in oxidation-reduction reactions catalyzed by dehydrogenases. Besides, there is no evidence that pyridine nucleotides occur naturally in the α-configuration. In the last three decades a large number of dehydrogenases that require NAD^+ or $NADP^+$ or both have been found in plants, animals and microorganisms. Figure 1 shows the structure of NAD^+. NAD^+ is probably the most abundant coenzyme in nature and functions as an electron carrier in both aerobic and anaerobic oxidation-reduction systems. The importance of coenzymes in enzyme catalyzed reactions is well known (75). For instance, 52% of the enzymes classified in 1979 by the International Union of Biochemistry require a coenzyme for activity. Handler made the observation that hydrolytic reactions differed from coenzyme-dependent reactions in that water rather than a coenzyme was the acceptor in the reaction (60). Of the over 2100 enzymes known up to 1979, 360 require pyridine nucleotides as a coenzyme, representing 17% of the total.

A general overview of the anabolism of NAD^+ in eukaryotic organisms is shown in figure 2. Two pathways for the synthesis of NAD^+ are known although, they do not operate in all eukaryotic systems. De novo synthesis of NAD^+ involves the conversion of tryptophan to quinolinic acid

Figure 1. The chemical structure of NAD^+.



NAD+

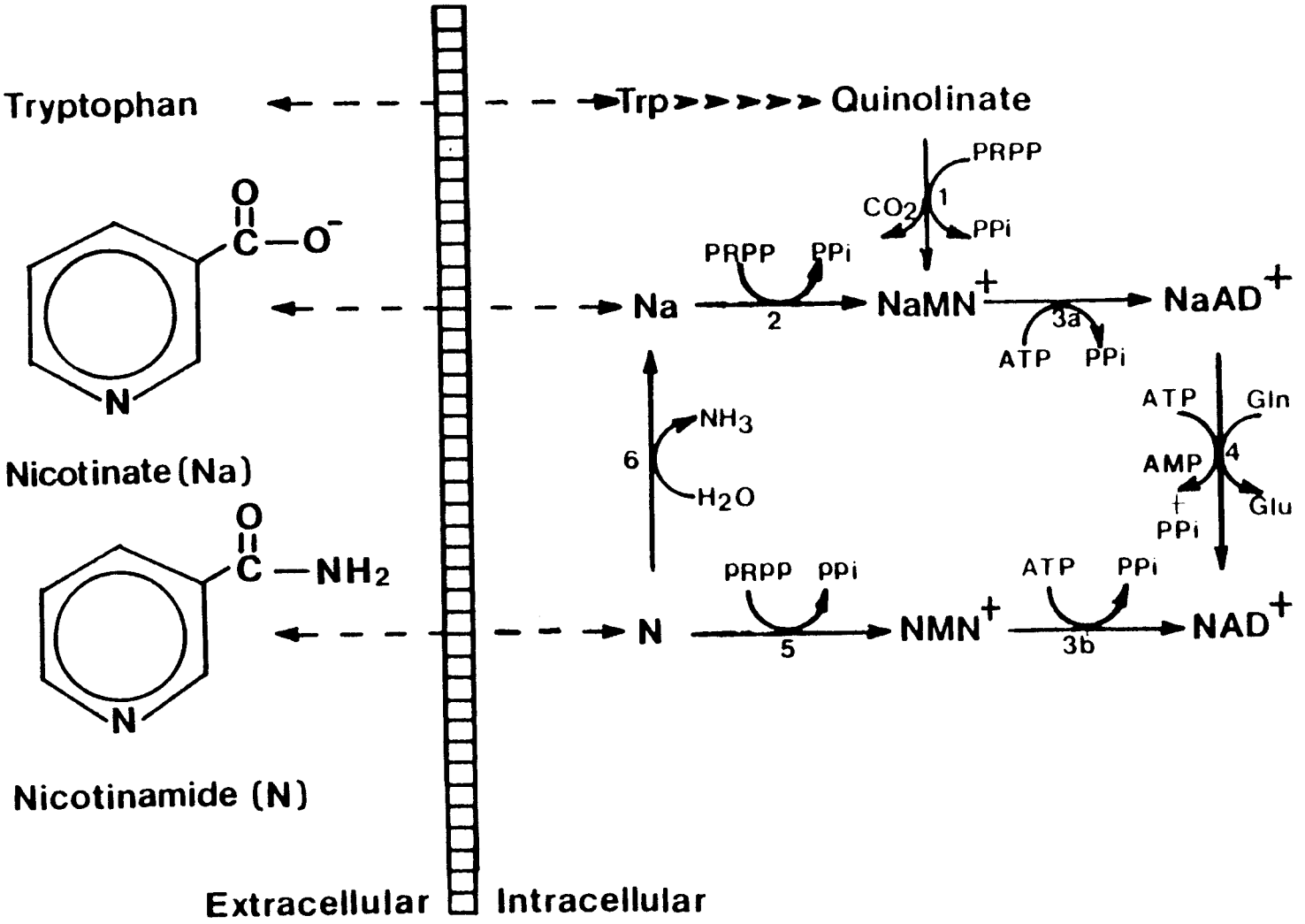
which in turn can be converted to NAD^+ via nicotinic acid mononucleotide by the enzymes quinolinate phosphoribosyl-transferase, NMN^+:adenyl transferase and glutamine hydrolyzing-NAD^+ synthetase. The salvage pathways involve the utilization of preformed pyridine rings, either nicotinamide or nicotinate. The enzymes responsible for this are nicotinamide phosphoribosyl transferase and nicotinate phosphoribosyl transferase, respectively.

Quinolinate phosphoribosyl transferase was first described by Nishizuka, et al. (132). However, it has only been found in hog liver and kidney (53). NMN^+:adenyl transferase was first found by Kornberg (100) in lower eukaryotes and subsequently has also been found in higher eukaryotes (46). In contrast, NAD^+ synthetase has only been found in mammalian tissues such as rat liver (143). Since these enzymes are not synthesized in all mammalian tissues, the salvage pathways for the synthesis of NAD^+ function as the major source of NAD^+ in higher eukaryotes.

The Catabolism of Nicotinamide Adenine Dinucleotide

Although the biological function of the pyridine nucleotides as electron transferring molecules is essential for virtually all forms of life, this is not the only metabolic role of these biomolecules. For instance, NAD^+ can be used as a substrate by several other classes of

Figure 2. Metabolic pathways for the synthesis of NAD^+ in eukaryotic organisms. (1) Quinolinate phosphoribosyl transferase. (2) Nicotinate phosphoribosyl transferase. (3a,3b) NaMN:adenyl transferase and NMN:adenyl transferase. (4) glutamine hydrolizing NAD^+ synthetase. (5) Nicotinamide phosphoribosyl transferase. (6) Nicotinamidase.


Tryptophan
Trp
Quinolinate
PRPP
CO2
1
PPi
PRPP
PPi
Na
2
NaMN+
3a
NaAD+
ATP
PPi
NH3
6
H2O
ATP
Gln
AMP
4
Glu
PPi
Nicotinate (Na)
PRPP
PPi
ATP
PPi
N
5
NMN+
3b
NAD+
Nicotinamide (N)
Extracellular
Intracellular

enzymes which consume NAD^+ by taking advantage of the fact that it contains two linkages that posses a high negative free energy of hydrolysis. For instance, the phospho-anhydride linkage of NAD^+ is used as the energy source when cleaved to yield nicotinamide mononucleotide (NMN^+) and AMP in E. coli and other prokaryotes during the synthesis of phosphodiester linkages of DNA, catalyzed by DNA ligase (138,187). In an equivalent reaction that takes place in eukaryotes, ATP is used as the source of energy instead. The N-glycosylic linkage of NAD^+ is also considered a high energy bond since its standard free energy of hydrolysis is approximately -8.2 kcal/mol (186). The energy released by the cleavage of this bond supplies the thermodynamic driving force for the covalent modification of proteins by ADP-ribosylation which is the subject of this work. However, before dealing with this topic in detail, the catabolism of NAD^+ will be considered.

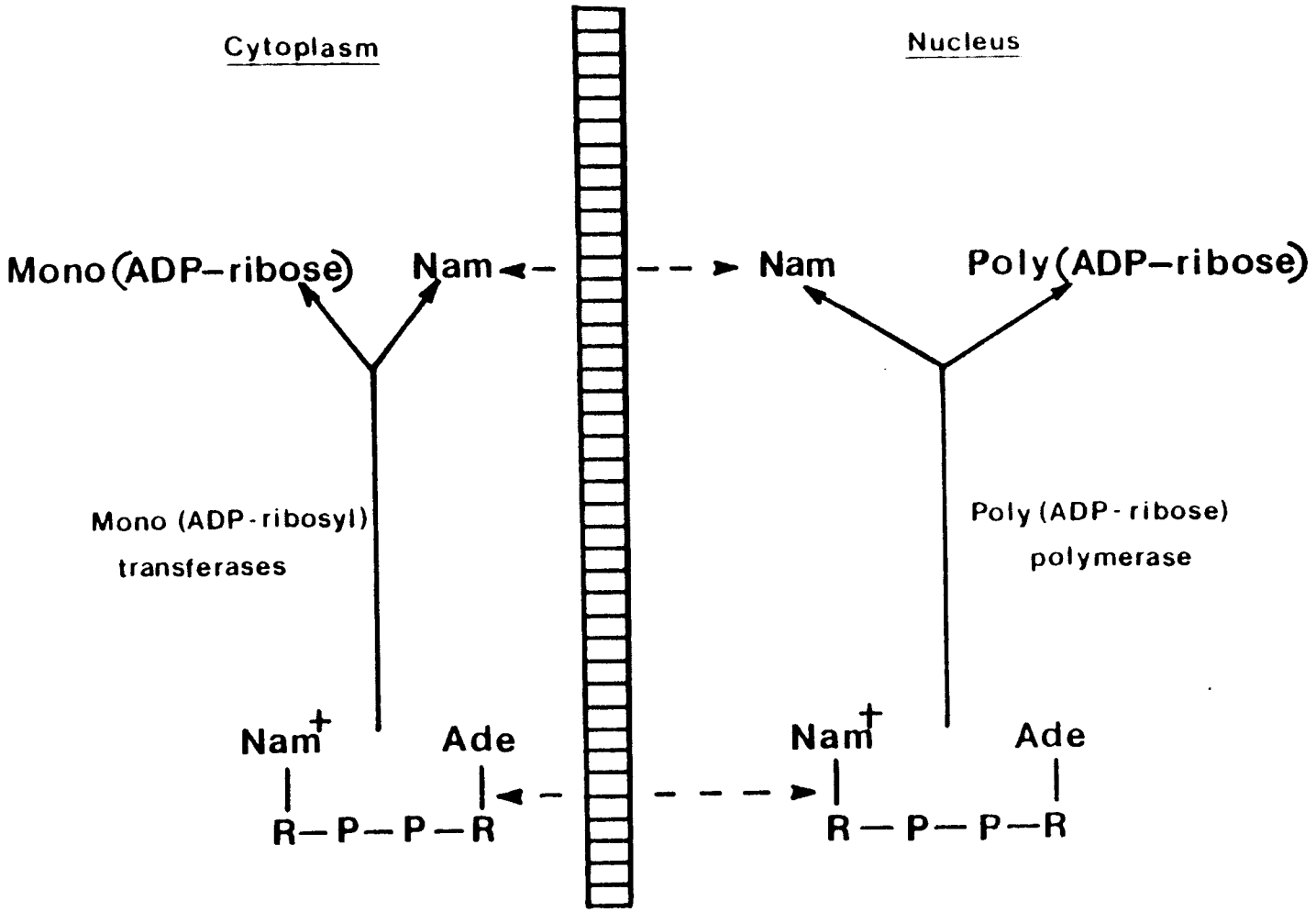
Most of the degradation of NAD^+ takes place by the hydrolysis of the N-glycosylic linkage, which yields ADP-ribose (adenosine diphosphate-ribose) with the concomitant release of nicotinamide (61,94), which is recycled into NAD^+ by the salvage pathway shown in figure 2. There are two different general classes of enzymes that catalyze the hydrolysis of NAD^+ into ADP-ribose and nicotinamide. Figure 3 shows the degradation pathways of NAD^+. One of these classes is the NAD^+ glycohydrolases which transfer the ADP-

ribose moiety to water and the other is the ADP-ribosyl transferases that modify proteins by ADP-ribosylation in a post-translationally. The ADP-ribosyl transferases may be further subclassified into mono(ADP-ribosyl) transferases and poly(ADP-ribose) polymerase. It is important to note that while mono(ADP-ribosylation) reactions have been shown to occur in prokaryotes and eukaryotes the synthesis of poly(ADP-ribose) seems to be unique to eukaryotes.

Mono(ADP-ribose) Metabolism

Mono(ADP-ribosyl) transferases (reviewed in Refs. 36, 54,127,166,171) catalyze the covalent modification of proteins at the specific amino acid side chains of arginine (123,125), asparagine (110) or diphtamide (73,170). The latter one is a hypermodified histidine residue found in elongation factor 2 (EF2), in all eukaryotic sources examined, including plants (26). ADP-ribosylation of EF2 by the bacterial toxin of Corynebacterium diphteriae with the resulting inactivation of EF2 and inhibition of protein synthesis was the first documented example of this type of covalent modification (35,69). In the last 15 years several other transferases that mono(ADP-ribosylate) eukaryotic proteins have been identified in prokaryotic systems. These include the bacterial toxin of Pseudomonas aeroginosa which also modifies EF2 at the diphtamide residue (72) as well as

Figure 3. Metabolic pathways for the degradation of NAD^+ in eukaryotic systems.


Cytoplasm
Nucleus
Mono(ADP–ribose)
Nam
Nam
Poly(ADP–ribose)
Mono (ADP-ribosyl)
transferases
Poly (ADP-ribose)
polymerase
Nam+
Ade
Nam+
Ade
R—P—P—R
R—P—P—R

the bacterial toxins of Vibrio cholerae (54,55), Bordetella pertussis (95,96) and Escherichia coli (127) which stimulate adenylate cyclase activity by mono(ADP-ribosylation) of the stimulatory or inhibitory GTP binding proteins that regulate the synthesis of cyclic AMP from ATP. A review of the role of the GTP binding proteins in the regulation of adenylate cyclase activity by the adrenergic hormones and the relationship with mono(ADP-ribosylation) has recently been presented by D. M. Payne (140). In addition, it has also been shown that transducin, a component of rhodopsin in the outer segments of the retinal rod is mono(ADP-ribosylated) by the toxins of Bordetella pertussis and Vibrio Cholerae (1,56,169). It is interesting to note that the polypeptide chains of transducin are very similar to the components of the GTP binding proteins of the adenylate cyclase complex.

Endogenous mono(ADP-ribosyl) transferases have also been found in eukaryotic systems. For example, four different activities have been reported in erythrocytes of variable sources by Moss and co-workers (124,126,128,129, 184). Some more have been described in rat, bovine and hen livers respectively (124,146,162). An endogenous enzyme that modifies EF2 in vitro has also recently been reported (105). It is important to note that Smith, et al. (153) and Hayaishi and co-workers (136) have been able to detect enzyme activities that remove protein bound monomeric ADP-ribose. These results provide strong evidence that the

covalent modification of proteins to regulate enzyme activities via mono(ADP-ribosylation), is a reversible process in a manner analogous to other post-translational covalent modifications such as phosphorylation or acetylation. It is important to note that while it has been demonstrated that some bacterial toxins alter the cellular metabolism of the host by this type of modification the physiological function of endogenous mono(ADP-ribosylation) reactions in eukaryotes is very poorly understood.

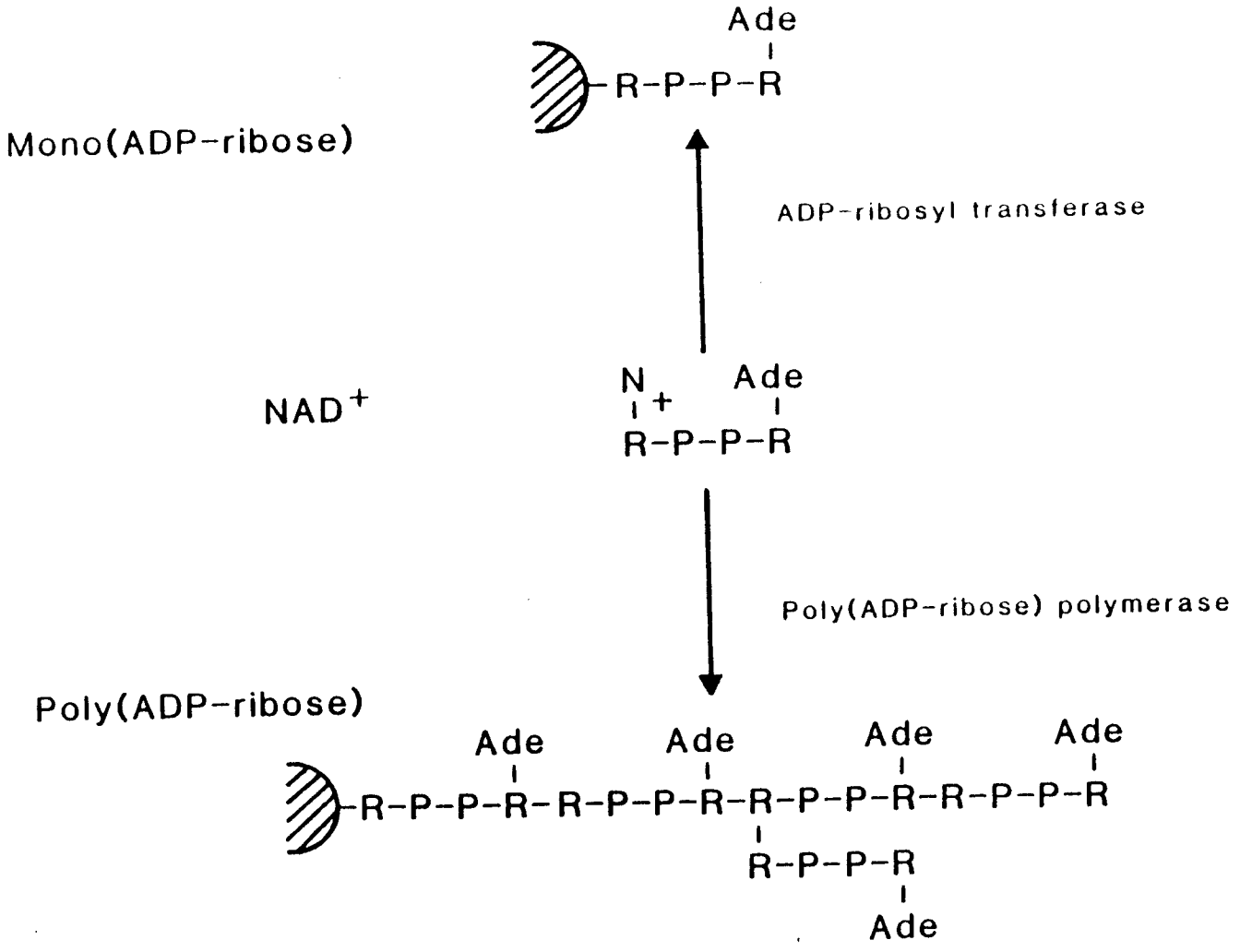
Poly(ADP-ribose) Metabolism

The work to be presented in this dissertation directly involves studies of poly(ADP-ribose) (for reviews see Refs. 65-67,145,109,156). Therefore, a more detailed discussion of the metabolism and possible biological function(s) of the polymer, will be presented. The history of poly(ADP-ribose) goes back to 1963 when Chambon, et al. (30) observed that the incorporation of [Ade-^{14}C] ATP into the acid insoluble fraction was stimulated about 1000-fold in the presence of NMN^+ while studying the synthesis of poly (adenylic acid) [Poly (A)] catalyzed by hen liver nuclei. This observation was corroborated and extended by Fujimura, et al. (50,51). They showed that the acid insoluble molecule obtained by Chambon, et al. could not possibly be poly (A) because this compound yielded very little AMP after enzyme digestion with

phosphodiesterase, and also because this acid insoluble molecule was stable upon treatment with 0.5 N NaOH at 37°C for 3 hours. In the late 1960's, these two groups of investigators as well as Hayaishi and co-workers obtained evidence that indicated that the novel compound observed was a homopolymer of ADP-ribose units linked together by ribose (1"-2') ribose α-glycosidic linkages. In 1979, Miwa, et al. demonstrated that poly(ADP-ribose) synthesized from nuclei from calf thymus was a branched molecule and suggested a branching frequency of one in every 40 to 50 residues of ADP-ribose of high molecular weight polymers (121). In 1979, Jacobson and co-workers conclusively demonstrated the existence of poly(ADP-ribose) in vivo using for the first time a reliable method to measure the total polymer levels (150). In 1982 Juarez-Salinas, et al. presented evidence of the occurrence of branched polymers of ADP-ribose in nucleotide permeable cells using this method (91). In addition, they also demonstrated the occurrence of branched polymers of ADP-ribose in intact cells (90). To date, poly(ADP-ribose) appears to be a polymer found uniquely in the nuclei of eukaryotic cells. Figure 4 shows an abbreviated representation of NAD^+ and its use as an enzyme substrate for the covalent modification of a protein by either mono(ADP-ribosylation) or poly(ADP-ribosylation).

Poly(ADP-ribose) is synthesized from NAD^+ in the nuclei of eukaryotic cells in a chromatin associated event.

Figure 4. Abbreviated form of the structure of NAD^+ and its use as a substrate for the covalent postranslational modification of a protein by mono(ADP-ribosylation) or poly(ADP-ribosylation).


Ade
R-P-P-R
Mono(ADP-ribose)
ADP-ribosyl transferase
NAD+
N+
Ade
R-P-P-R
Poly(ADP-ribose) polymerase
Poly(ADP-ribose)
Ade
Ade
Ade
Ade
R-P-P-R-R-P-P-R-R-P-P-R-R-P-P-R
R-P-P-R
Ade

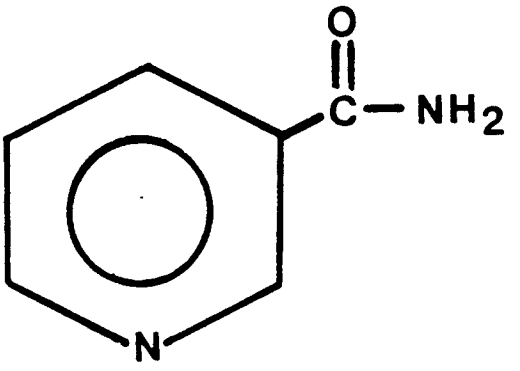
This enzyme has been characterized from many sources and has been highly purified (76,93,131,167,182). The enzyme requires DNA as an assential activator. Magnesium ions and histones as well as other positively charged proteins such as protamines stimulate the activity of the purified enzyme. It has recently been reported that poly(ADP-ribose) polymerase is a metallo-enzyme, because of its tight association with zinc (185). In addition, it has also been reported that the enzyme is inhibited by zinc in vitro (104). The synthesis of poly(ADP-ribose) is inhibited by a spectrum of inhibitors (168). Table I shows the major different classes of inhibitors of poly(ADP-ribosylation) that have been used. These include, nicotinamide and its analogues (144), the methylxanthines and the adenine containing nucleotides as well as others (106). Figure 5 shows the chemical structure of the most widely used inhibitors, the nicotinamide analogues. These include benzamide (BA), 3,amino-benzamide (ABA) and 3,methoxy-benzamide (MBA). The respective nicotinate analogues are non-inhibitory. An important point is that the nicotinamide analogues as well as other inhibitors of poly(ADP-ribose) synthesis are also inhibitors of mono(ADP-ribosyl) transferases and therefore interpretations of the effects observed by the use of these inhibitors in intact cells should be made with caution. In addition, these compounds

TABLE I

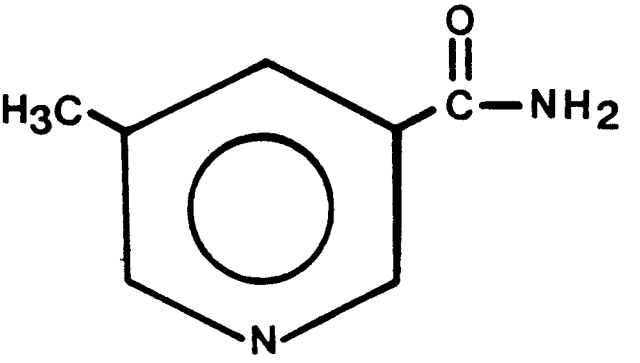
Inhibitors Of Nuclear Poly(ADP-ribose) Polymerase

Nicotinamide analogues	Methylxanthines	Others
Nicotinamide	Theophylline	Thymidine
5,Methyl-nicotinamide	Theobromine	Thymine
Benzamide	caffeine	Ap_4A
3,Amino-benzamide		Ap_5A
3,Methoxy-benzamide		Ap_6A
		α-NAD^+
		NADH

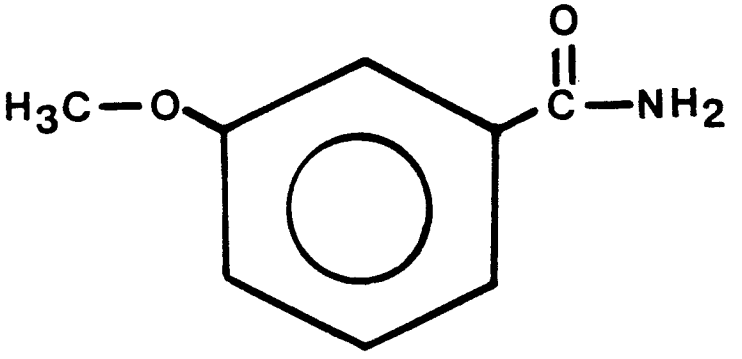
Figure 5. Chemical structures of the nicotinamide analogues used as inhibitors of poly(ADP-ribosylation) reactions.



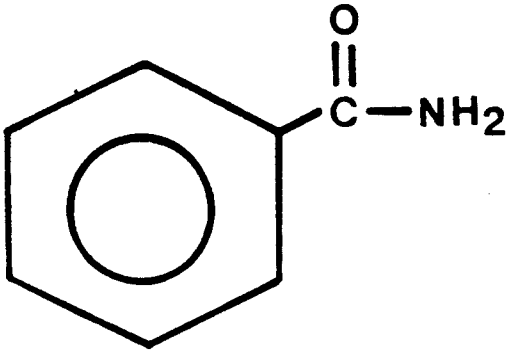
a) Nicotinamide



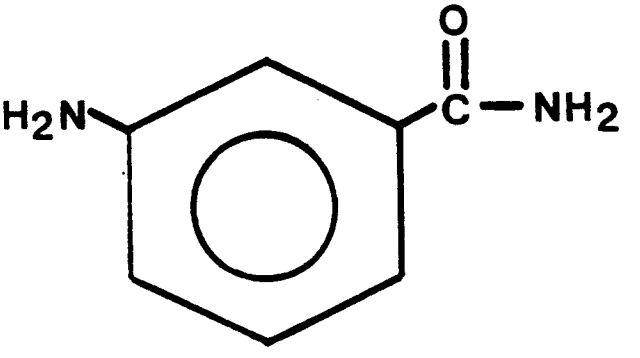
b) 5—Methylnicotinamide



c) 3—Methoxybenzamide



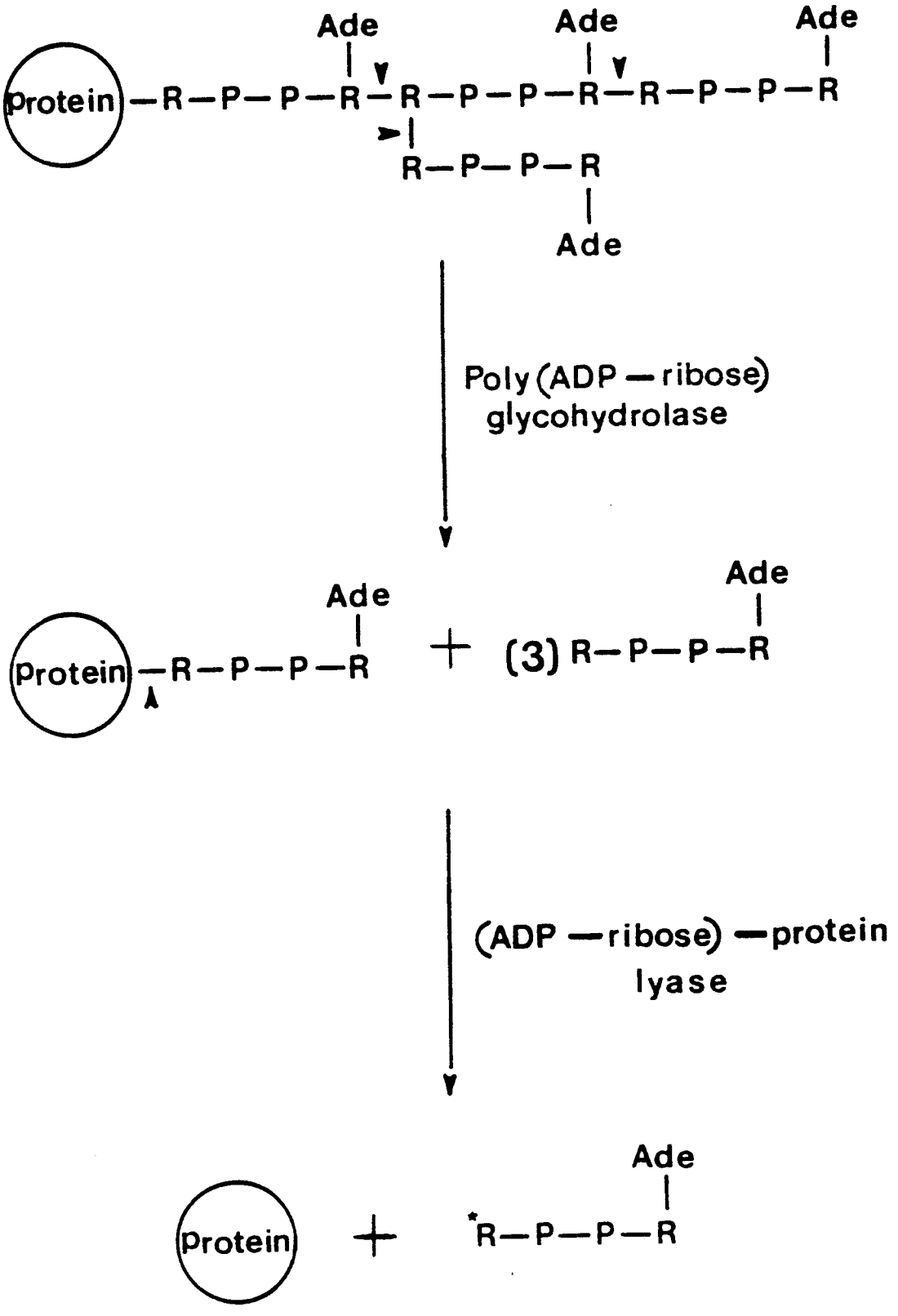
d) Benzamide



e) 3—Aminobenzamide

inhibit purine synthesis at concentrations above 5 mM (32). These concentrations also cause alterations of the overall metabolic rate of cells (112). Nevertheless, it is important to note that these inhibitors do not significantly inhibit poly(ADP-ribose) glycohydrolase and/or NAD^+ glycohydrolase. Polymers of ADP-ribose are degraded by two different kinds of enzymes, poly(ADP-ribose) glycohydrolase (117,118) and phosphodiesterases (52,122). The former enzyme appears to be responsible for the turnover of the polymer in vivo based on its affinity and specificity for poly(ADP-ribose) (120). This enzyme catalyzes the hydrolysis of the o-glycosidic linkages of the polymer to generate monomeric ADP-ribose. This activity has been shown to be inhibited by ADP-ribose, cyclic AMP and Ca^{++} (165). The residual protein-bound monomeric ADP-ribose is not a substrate for the glycohydrolase and is released from protein by a protein-(ADP-ribose) lyase (136,137,153). Figure 6 shows the metabolic pathway for the degradation of poly(ADP-ribose) in vivo. In contrast, phosphodiesterases appear not to play a major role in the degradation of the polymer in vivo. Nevertheless, they have been very useful in studying poly(ADP-ribose) in vitro. For example, snake venom phosphodiesterase (SVPD) has been widely used for the generation of the unique nucleotide phosphoribosyl adenosine monophosphate (PRAMP), also called iso-(ADP-ribose), by the cleavage of linear internal phosphoanhydride bonds of the

Figure 6. Metabolic pathway for the degradation of poly(ADP-ribose) *in vivo*. (*) Chemically modified mono(ADP-ribose).


Ade
Ade
Ade
protein
—R—P—P—R—R—P—P—R—R—P—P—R
R—P—P—R
Ade
Poly (ADP — ribose)
glycohydrolase
Ade
Ade
protein
—R—P—P—R
+ (3) R—P—P—R
(ADP —ribose) —protein
lyase
Ade
protein
+
*R—P—P—R

polymer. The non-reducing end of the polymer gives rise to 5'-AMP which can be used in conjunction with PRAMP to estimate the chain length of linear polymers. This has been the rationale followed by several groups to determine the average size of polymers of ADP-ribose synthesized *in vitro* (4,117,159). However, because poly(ADP-ribose) can be a branched molecule, the number of AMP residues obtained per molecule can be more than one, depending on the number of branching points per molecule. This can lead to an underestimation of the polymer size. A branched polymer of ADP-ribose digested with SVPD will generate diphospho-diribosyl adenosine monophosphate [$(PR)_2AMP$] which can be used as a reference standard to detect points of branching in polymers of ADP-ribose *in vivo* (91). The use of SVPD in combination with bacterial alkaline phosphatase was also important in the development of a highly specific and sensitive assay to measure total polymer levels in intact cells (85).

Covalent Acceptors for Poly(ADP-ribosylation) in vitro

Histone H1 (29,133,142,147) and the poly(ADP-ribose) polymerase itself (97,157,181) are major covalent acceptors for poly ADP-ribosylation *in vitro*. It has been shown that automodification of the enzyme leads to its inactivation (19). In addition, it has been shown that other proteins

can also serve as acceptors of poly(ADP-ribose) chains as well. These include, a Ca^{++},Mg^{++} endonuclease (183), the high mobility group proteins (HMG's), core histones (141) and topoisomerase I (47). However, it is important to point out that the role of of the covalent modification of these proteins by poly(ADP-ribosylation) in vivo, remains to be established. In addition, it is of interest to note that a dinucleotide such a Ap_4A inhibits the poly(ADP-ribosylation) of histone H1 and it may serve as an acceptor for poly(ADP-ribosylation) in vitro as well (160,161,180). Most of the protein-poly(ADP-ribose) conjugates formed in vitro are linked via carboxylate ester linkages to either glutamic acid residues or carboxy terminal lysine residues (27). This bond is very labile under even mild alkaline conditions. It has also been suggested that the bonds between poly(ADP-ribose) and proteins are heterogeneous because of their different chemical stability. A fraction of the conjugates are not susceptible to cleavage by neutral hydroxylamine or dilute alkali (67).

Poly(ADP-ribose) Metabolism and DNA Excision Repair

Several attempts have been made to elucidate the physiological function(s) of poly(ADP-ribose). It is reasonable to expect changes in chromatin structure due to the metabolism of the polymer within chromatin. Current

data indicate that there is a very "close relationship" between poly(ADP-ribose) metabolism and DNA excision repair mechanisms and related processes. Evidence that supports this relationship comes from the fact that the activity of the poly(ADP-ribose) polymerase increases dramatically with the formation of DNA strand breaks (16,22,23,58,86). The requirement of the enzyme for nicked DNA was first suggested by Janakidevi and Koh (86). However, the possible significance of DNA fragmentation on poly(ADP-ribose) metabolism was first recognized by Miller (114), when he was able to demonstrate that poly(ADP-ribose) polymerase activity measured in vitro in the presence of DNase I was substantially increased. Subsequent experiments have shown that the polymerase activity is also stimulated by other nucleases (113). Thus, Benjamin and Gill (14,15) were the first to correlate quantitatively polymerase activity with DNA strand breaks. It is interesting to note that different types of DNA strand breaks stimulate the synthesis of poly(ADP-ribose) to differing degrees. They showed that double stranded restriction fragments of DNA with flush ends are the most effective in the stimulation of the polymerase. This kind of DNA proved to be about three times more efficient than DNA fragments that contained nucleotides extending from the 3' end and it was about ten fold better than fragments containing unpaired nucleotides at the 5' end. Early studies on rates of poly(ADP-ribose) synthesis

with isolated nuclei from cells under different conditions should be interpreted with caution. The fluctuations observed in enzyme activity could be due to the formation of DNA strand breaks during the isolation of nuclei. Thus, results obtained using *in vitro* systems should not be extrapolated to the function of the polymer *in vivo*. Therefore, recent attempts to understand the function of the polymer have been focussed on *in vivo* studies. It has been known for some time that the intracellular levels of NAD^+ are depleted following treatment with different carcinogenic agents (17,63,68,174). No reasonable explanation for these observations was available until it was shown by Jacobson and co-workers that the drop in the NAD^+ pools was due to the activation of poly(ADP-ribose) polymerase and not due to inhibition of NAD^+ biosynthesis or activation of other enzymes that consume NAD^+ such as NAD^+ glycohydrolase,NAD^+ pyrophosphatase or NAD^+ kinase (84). Thus, these observations provided a clue to the relationship between poly(ADP-ribose) metabolism and the cytotoxic effects of alkylating agents. However, the amounts of poly(ADP-ribose) observed following DNA damage did not account for the total drop of the NAD^+ pools. Thus, it was reasonable to suggest that synthesis and turnover of the polymer were very closely coupled and that the turnover of poly(ADP-ribose) should be relatively rapid. Initial evidence that this was the case was presented by Juarez-Salinas *et al.* (92) when they showed

that following treatment of intact cells with N-methyl,-N'-nitro,N-nitrosoguanidine (MNNG), a potent carcinogenic agent that induces DNA strand breaks, the poly(ADP-ribose) intracellular levels increased more than 100 fold and turned over rapidly. It has also been shown that the depletion of the NAD^+ pools (39,111), as well as the accumulation of the poly(ADP-ribose) (177) can be effectively blocked by the use of inhibitors of poly(ADP-ribose) synthesis. Moreover, it has been demonstrated by studies with the aid of inhibitors that the half-life of poly(ADP-ribose) is approximately 6 minutes or less in human cells following UV treatment (77) and less than 1 minute in mouse fibroblasts following MNNG treatment (83).

Other available data also support a relationship between poly(ADP-ribose) metabolism and DNA excision repair. This information has been obtained using different classes of DNA damaging agents. These include treatments with x-rays (14), UV-irradiation (34), gamma-irradiation (152) and alkylating agents such as N-methyl-N'-nitroso urea (MNU) (130), dimethyl sulfate (DMS) (41) and MNNG (83,92). It is important to note that cytotoxic agents that do not have any effects on the integrity of DNA also failed to influence the intracellular levels of NAD^+ or the metabolism of poly(ADP-ribose). In addition, one must keep in mind that most of the studies done with alkylating agents use concentrations very much above what is considered to be a lethal dose.

Therefore, conclusions about the observations previously described, should be drawn with caution.

It has been demonstrated that inhibitors of poly(ADP-ribose) synthesis at non-cytotoxic concentrations potentiate the cytotoxicity of alkylating agents (20,42,179). These results suggest that poly(ADP-ribosylation) and chromatin associated events are necessary for cells to recover from treatment with alkylating agents. These data provide very strong evidence in favor of the participation of poly(ADP-ribose) in DNA excision repair. Most of the published data indicate that changes in the metabolism of poly(ADP-ribose) are coincident with DNA excision repair (42,77). However, although poly(ADP-ribose) metabolism and DNA excision repair mechanisms are required for cellular recovery from DNA damage, there is the possibility that there is no connection between the two processes.

DNA excision repair may be divided into four steps: 1).- Incision near the lesion within the DNA, 2).- removal of modified or damaged nucleotides, 3).- DNA resynthesis and 4).- Ligation of the DNA nicks. Current data demonstrate that the rate of removal of DNA lesions observed during unscheduled DNA synthesis in the presence of poly(ADP-ribose) polymerase inhibitors, is either unchanged or slightly increased (33,43). With regard to DNA repair replication either no effect (33,77) or a slight stimulation was observed (6,18,43,119,151). In addition, Durkacz et al.

(42) demonstrated that when DMS was used to induce DNA strand breaks, the DNA was of the same size as undamaged DNA, as estimated by alkaline sucrose gradients, following the removal of DMS. If ABA was included in the medium the steady state number of DNA strand breaks was considerably increased. Thus, Shall and co-workers (37) have suggested that poly(ADP-ribose) synthesis is important in the ligation step of DNA excision repair. They have also reported that the activity of DNA ligase II is regulated by poly(ADP-ribosylation), although they did not show whether or not the enzyme itself was covalently modified by ADP-ribosylation (37). However, consolidation of this hypothesis awaits further evidence. Moreover, the data available at present are also consistent with a role of poly(ADP-ribose) at the incision step of DNA excision repair.

Poly(ADP-ribose) Metabolism and Other Cellular Responses to DNA damage

Several studies have attempted to examine the possible role of poly(ADP-ribose) metabolism in other biological responses to DNA damage. These include cellular mechanisms related to carcinogenesis such as mutagenesis and malignant transformation. These biological responses are interrelated with DNA excision repair, but these relationships are poorly understood. In addition, it is important to note that these

cellular responses to DNA damage require DNA replication and cell division for expression. Thus, Jacobson et al. as well as others (38,79,98) have shown that in dividing cells inhibitors of ADP-ribosylation alter S phase and block cell division following DNA damage. Therefore, studies suggesting that mutation frequency (24,149) and cellular transformation (25,103,107) are altered in the presence of inhibitors of the synthesis of poly(ADP-ribose) following DNA damage should be interpreted with caution. Initial experiments done by Jacobson et al. (81) showed that when using non-lethal doses of MNNG in combination with MBA in C3H10T1/2 cells in culture, a dramatic increase in the the rate of malignant transformation was observed while the mutation frequency was partially inhibited. Moreover, they did not succeed in detecting any effect of the inhibitors of poly(ADP-ribose) synthesis during potentially lethal damage repair when using quiescent cells. Thus, the effects of the inhibitors of poly(ADP-ribose) synthesis on the rate of mutagenesis and malignant transformation suggest that poly(ADP-ribose) metabolism may in fact be related to carcinogenesis, although, it is possible that there is no relationship between the two processes.

Poly(ADP-ribose) Metabolism and Gene Expression

Gene expression requires chromatin reorganization therefore, it is possible that poly(ADP-ribose) metabolism plays a role in this biological phenomenon as well. Evidence supporting this notion comes from several studies that relate poly(ADP-ribose) metabolism to cellular phenomena involving gene rearrangements. For instance, nicotinamide analogues affect cellular differentiation in different systems (5,45,88). Further evidence that poly(ADP-ribose) is required for proper gene expression has been presented by Tanuma and Johnson (163). They observed that poly(ADP-ribosylation) of HMG proteins was required for proper gene expression in mouse mammary tumor cells treated with glucocorticoids (163,164). The inhibition of the poly(ADP-ribosylation) of HMG's 14 and 17 in the presence of ABA prevented proper gene expression. In addition, nutritional depletion of NAD^+ and the inhibitors of poly(ADP-ribosylation) have been shown to stimulate sister chromatid exchanges (SCE's) and non-homologous interchanges (70,134). It has recently been proposed by Juarez-Salinas, et al. (89) that poly(ADP-ribose) might be involved in the cellular responses to stress, because of the changes observed in poly(ADP-ribose) metabolism following treatment of cells with hyperthermia and other cellular stress inducing agents. It is known that hyperthermia elicits the

synthesis of a specific set of proteins in a number of systems (10). These proteins are referred to as they are synthesized while most of transcription and protein synthesis are turned off. Therefore, poly(ADP-ribose) appears to be also required by cells to adapt to changes in the environment.

Research Prospectus

Despite a large body of available information, we still do not fully understand the biological function(s) of poly(ADP-ribose). The regulation of chromatin functions by poly (ADP-ribosylation) reactions might be achieved by two general types of mechanisms. First, it is possible that regulation of enzyme activities in chromatin is affected by the covalent modification of nuclear proteins via poly(ADP-ribosylation). Second, non-covalent interactions between this negatively charged molecule and other chromatin components may play a key role. The evaluation of the relative contributions of these two different factors require knowledge of the nature of the covalent acceptors *in vivo* as well as information of the complexity of the polymer. Recent studies have provided evidence which suggest that histone H2b and possibly the polymerase itself are poly(ADP-ribosylated) *in vivo* following DNA damage (3,102). In addition, Wong *et al.* have proposed that two

histone H1 molecules are linked together by an oligo(ADP-ribose) molecule of 15 to 16 residues (178) *in vivo*. Finally, the HMG proteins have also been shown to be modified *in vivo* by poly(ADP-ribosylation) (163). It is clear that our understanding of the covalent functions of poly(ADP-ribose) in chromatin structure *in vivo*, is still very limited. Only limited information about the size of poly(ADP-ribose) synthesized *in vitro* has been reported and no information concerning the complexity of the polymer *in vivo* has appeared in the literature. Thus, this dissertation evaluates the potential importance of non-covalent interactions of poly(ADP-ribose) with other chromatin components under different *in vivo* conditions.

CHAPTER II

MATERIALS

Biologicals

Balb/c SV40 virus-transformed 3T3 mouse embryo fibroblasts (SVT2) were obtained from Dr. George Todaro, National Cancer Institute (Bethesda, MD). C3H10T1/2 (clone 8) cells were obtained from Dr. C. Heidelberger, Comprehensive Cancer Center, Univeristy of Southern California (Los Angeles, CA). Dulbecco's modified Eagle's medium and an antibiotic-antimycotic mixture (100x) containing 10,000 units/ml of penicillin, 10,000 units/ml of streptomycin and 25 µg/ml of fungizone were purchased from Grand Island Biological Company (Grand Island, NY). Fetal calf serum was obtained from Reheis Chemical Company (Kankakee, IL). Gentamycin was obtained from Schering (Kenworth, NJ) and the supplier of amphotericin B was Sigma Chemical Company (St. Louis, MO).

Radioisotopes

Alpha-[^{32}P] adenosine triphosphate (1000-2000 Ci/mmol) and [2-8,^{3}H] adenine (29 Ci/mmol) were obtained from

International Chemical and Nuclear (Irvine, CA). Aquasol and rapid autoradiographic enhancer (Enlightning solution) were purchased from new England Nuclear (Boston, MA). Screens for autoradiography intensifying were obtained from E.I. Dupont De Nemours & Company (Wilmington, DE).

Enzymes

Alkaline phosphatase (E. coli) type II-S, deoxyribonuclease I from beef pancreas (DNase I), proteinase K (Tritrachium album) type IX, pronase E (Streptomyces griseum) type XIV, and trypsin were purchased from Sigma Chemical Company (St. Louis, MO). Snake venom phosphodiesterase was obtained from Worthington Biochemical Corporation (Freehold, NJ). NMN^+:adenyl transferase (Nicotinamide adenine dinucleotide pyrophosphorylase) (hog liver) was obtained from Boehringer Mannheim (Indianapolis, IN).

General Supplies

Adenosine 5'-monophosphate (AMP), Adenosine 5'-diphosphate (ADP), Adenosine 5'-triphosphate (ATP), Nicotinamide adenine dinucleotide (NAD^+), tris(hidroxymethyl)-aminomethane (Tris), Triton X-100, Guanidine hydrochloride (Gu.HCl), Nicotinamide mononucleotide (NMN^+)

4-morpholinepropanesulfonic acid (MOPS) and 1-ethyl, 3(3-dimethyl-aminopropyl) carbodiimide hydrochloride (EDC) were purchased from Sigma Chemical Company (St. Louis, MO). The supplier for 6-aminohexanoic acid and m-aminophenylboronate hemisulfate was Aldrich Chemical Company (Milwaukee, WS). Bio Rex 70 (200-400 mesh size), a cation exchange resin; Affi-gel 601, a boronate gel; Blue Sepharose; Acrylamide; N,N'methylene-bis-acrylamide; N,N'-bis-acrylcystamine; ammonium persulfate; N,N,N',N' tetramethylene-diamine (TEMED); Bromophenol blue (BPB); polypropylene Econo-Columns (0.8 cm I.D.) and the Bio-Sil TSK-125 columns were obtained from Bio Rad (Richmond, CA). Homopolymers of adenylic acid of different size were purchased from P.L. Biochemicals (Milwaukee, WS). The Partisil 10-SAX column (250 mm x 4.5 mm I.D.) was obtained from Whatman Chemical Separation, Incorporated (Clifton, NJ). For reversed-phase HPLC, an Altex ultrasphere-ODS column (250 mm x 4.6 mm I.D.) was obtained from Beckman Instruments (Palo Alto, CA). β-mercaptoethanol, xylene cyanol (XC), x-ray developer, x-ray fixer, BB-5 ray film of 8x18 and 10x14 inches were purchased from Eastman Kodak Company (Rochester, NY). Trichloroacetic acid (TCA), Ethylene-diamino-tetraacetate (EDTA), NaOH, $MgCl_2$, NaH_2PO_4, NaCl, KCl, NH_4HCO_3, $NaHCO_3$, KH_2PO_4, HCl, ammonium acetate (NH_4OAc), ammonium formate, and sodium acetate (NaOAc) were purchased from Fisher Scientific

(Fairlawn, NJ) and methanol HPLC grade was from Fisher Scientific (Dallas, TX). Diethyl ether and boric acid were obtained from MCB reagents (Cincinnati, OH). Matrex-gels PBA-10, PBA-30 and PBA-60 were provided by Amicon Corporation (Danvers, MA). The source of chloroacetaldehyde (45%) in water) was International Chemical and Nuclear (Plainview, NY). Mono-ADP-ribosylated histone H1 radiolabeled with [^{14}C] at the adenine moiety was kindly provided by Dr. J. Moss of the National Institutes of Health (Bethesda, MD). Bovine liver tyrosine-tRNA ($Q^{*}\psi A$), labeled with [^{32}P] was a gift of Dr. R.M. Pirtle of North Texas State University and was isolated as described elsewhere (87). Mono(ADP-ribose) labeled with [^{32}P] was made from NAD^{+} by Dr. D.M. Payne of North Texas State University as indicated in methods. Radiolabeled NAD^{+} was made from [α-^{32}P] ATP by Dr. R.C. Benjamin as indicated in methods.

CHAPTER III

METHODS

Synthesis of Radiolabeled Nicotinamide Adenine Dinucleotide

To synthesize radiolabeled NAD^+, [α-^{32}P] ATP was used as the substrate for NAD^+ pyrophosphorylase. The final incubation volume was 50 1 and contained 50 mM Tris-HCl, 30 mM phosphoenolpyruvate, 1.6 mM NMN^+, 2 mM $MgCl_2$, and the pH was adjusted to 7.5 with 1.0 N NaOH. The reaction mixture also contained 30 units of pyruvate kinase/ml, 0.3 units of NAD^+ pyrophosphorylase/ml and 5-7 µM [α-^{32}P] ATP (usually about 1 to 2 mCi). The solution was incubated at 37°C for 30 min. The reaction was quenched by diluting the sample to 10 ml using 0.25 M NH_4OAc_3 buffer, pH 9.0, and putting it on ice. The solution was chromatographed on a 0.5 ml DHB-Bio Rex column at room temperature. The column was washed with 15 ml of 0.25 M NH_4HCO_3 buffer, pH 9.0, and the radiolabeled NAD^+ was eluted with 4 ml of water. The eluate was frozen at -80°C and lyophilized to drynes. The material was redissolved in 3 ml of 10 mM sodium phosphate buffer, pH 6.0 and kept at -20°C until used. Normally, about 80% of the

total [^{32}P] ATP was incorporated into NAD^+ and the purity of the preparation was > 95% as judged by HPLC analysis.

Synthesis of Radiolabeled ADP-ribose

To synthesize [^{32}P] ADP-ribose, radiolabeled NAD^+ was incubated at 37°C for 30 min in 0.1 N NaOH in a final volume of 50-500 µl. The incubation mixture was diluted to 5 ml in 0.25 M NH_4Cl buffer, pH 9.0, the pH was adjusted to 9.0 and applied to a 0.5 ml column of DHB-Bio Rex. The column was washed with 0.25 M NH_4Cl buffer, pH 9.0 and the labeled ADP-ribose was eluted with 0.5 ml of 10 mM H_3PO_4, pH 2.3 containing 25 mM KCl. Routinely, about 90% of the radiolabeled NAD^+ was converted to radiolabeled ADP-ribose.

Synthesis of Dihydroxyboronyl-Bio Rex

Twenty five g of Bio Rex 70 (200-400 mesh size) were placed in a 500 ml flask and suspended in 300 ml of 0.1 M NH_4OAc buffer, pH 5.0. Two and a half g of m-aminophenyl-boronate hemisulfate and 2.5 g of EDC were suspended in 25 ml of 0.1 M NH_4OAc buffer, pH 5.0 and were added to the Bio Rex suspension. The pH was adjusted to 5.0 by the dropwise addition of 6N HCl, and the suspension was shaken at room temperature for 16 hours. The resin was then washed with 5 l of deionized water followed by 1 l of 0.1 M NH_4OAc buffer,

pH 4.5, containing 1 M NH_4Cl, followed by 1 l of NH_4HCO_3 buffer, pH 9.0, containing 1.0 M NH_4Cl and again with 3 l of deionized water. Finally, the resin was washed with 250 ml of 0.25 M NH_4OAc, buffer, pH 5.0, containing 6 M Gu.HCl and stored in the dark as a 1:1 suspension in the same buffer at 4°C.

Cell Permeabilization

SV40 virus-transformed Balb/c 3T3 mouse embryo fibroblasts (SVT2) were grown in a humidifier, 10% CO_2-air incubator at 37°C in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (heat inactivated, 60 min, 56°C), penicillin (100 units/ml), fungizone (25 µg/ml) and streptomycin (100 units/ml). Cells were seeded at approximately $8X10^5$ cells/75 cm^2 flask in 15 ml of medium. Cells were routinely fed every 48 hours until cell density reached 15 to $20X10^6$ cells/75 cm^2 flask. Flasks were removed from the incubator, washed twice with 10 ml of phosphate buffered saline (PBS) (0.01 M sodium phosphate buffer, pH 7.2, containing 150 mM NaCl). Cells were then removed from the flask by treatment with 2 ml/flask of 0.05% trypsin containing 200 mg/l of EDTA in the cold for 2 min. Treatment was terminated by the addition of 2 ml of medium and the cells were pooled in a centrifuge tube and the cell suspension was centrifuged at 1000xg for 20 min and the

pellet was kept frozen at -20°C. Cells were permeabilized by a slight modification of the method described by Berger et al. (21). The pellets were resuspended in permeabilization buffer in a final concentration of $2X10^6$ cells/ml and incubated for 15 min at 0°C. The buffer used contained 10 mM Tris-HCl, pH 7.8, 1mM EDTA, 30 mM β-mercaptoethanol, 2.5 mM $MgCl_2$ and 0.05% Triton X-100. The suspension was centrifuged at 1000xg, the supernatant was discarded and the pellet was resuspended in the incubation buffer at $2X10^7$ cells/ml. On light microscopic examination, the permeabilized cells appeared swollen but morphologically intact. An aliquot of the cell solution was mixed with an equal volume of trypan blue (0.4% solution in saline), and the percent of permeabilization was determined using an hemocytometer. More than 90% of the cells were rendered permeable, as demonstrated by the uptake of the dye.

Poly(ADP-ribose) Synthesis in Permeabilized Cells

The incubation mixture for poly(ADP-ribose) synthesis in permeabilized cells contained $2X10^7$ cells/ml, 40 mM Tris-HCl buffer, pH 7.8, 0.6 mM EDTA, 30 mM $MgCl_2$, 1 mM β-mercaptoethanol, 0.5 mM NAD^+, 100 µCi of [^{32}P] NAD^+ and 200 µg/ml of DNase I. The suspension was incubated for 30 min at 37°C and the reaction was terminated by adjusting to 20% TCA (w/v) using 100% TCA (w/v), and chilling on ice for

15 min. The pellet obtained by centrifugation at 10,000xg for 5 min was washed 5 times with 20% TCA, then three times with diethyl-ether and then dissolved in 2 ml of 0.1 or 0.2 N NaOH containing 20 mM EDTA by heating the sample at 60°C for 2 h. The solution was then neutralized and kept at -20°C until purification of the polymer. Poly(ADP-ribose) synthesis was determined by the rate of conversion of radiolabeled NAD^+ into acid insoluble material. A typical rate was approximately 20 pmol/min/10^6 cells.

Synthesis of Poly(ADP-ribose) in C3H10T1/2 Cells Following Treatment with MNNG

C3H10T1/2 cells were maintained in Corning plastic tissue culture flasks of 75 cm^2 and incubated in a humidified atmosphere of 10% CO_2 in air at 37°C. Cells were normally seeded at approximately 1.5X10^5 cells/flask. Cells were fed twice a week with Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal calf serum, 20 mg of gentamycin/l and 0.63 mg of anphotericyn B/l. Cultures were routinely subcultured (1:20 split) prior to reaching confluence, which represented about 2 to 3X10^6 cells/flask. Only cells from passage 20 or less were used. Cells were then labeled with 20 μCi/ml of [^{3}H] Ade in nicotinamide free Dulbecco's modified Eagle's medium containing 10% heat-inactivated dialyzed fetal calf serum

for 16 hours. Media was removed from the flasks followed by two rinses with PBS. Cells were then fed 10 ml of Dulbecco's modified Eagle's medium containing 10% heat-inactivated, dialyzed fetal calf serum previously equilibrated at 37°C and the cells were incubated at 37°C for 20 min following addition of MNNG to a final concentration of 64 µM. Medium was decanted and flasks were rinsed with cold PBS three times and 20% TCA (w/v) was added. Acid insoluble material was obtained by scraping the flasks followed by centrifugation at 1000xg for 10 min.

Synthesis of Poly(ADP-ribose) in C3H10T1/2 Cells Following Treatment with Hyperthermia and MNNG

C3H10T1/2 cells were grown to confluence and labeled as described above. Media was removed from the flasks, followed by two rinses with PBS. Cells were then fed with 10 ml of Dulbecco's modified Eagle's medium containing 10% heat-inactivated dialyzed fetal calf serum pre-equilibrated to 37°C containing 64 µM MNNG and cells were incubated at 37°C for 20 min. The medium was decanted, and flasks were rinsed three times with cold PBS, and 20% TCA was added. Acid insoluble material was obtained by scraping material from the flasks with a rubber policeman. The acid insoluble pools were obtained by centrifugation at 1,000xg for 10 min.

Synthesis of Poly(ADP-ribose) in SVT2 Cells Following Treatment with Heat Shock, MNNG and Ethanol

SVT2 cells were grown as described above and media was removed from the flasks followed by two rinses with PBS. Cells were then fed with 10 ml of Dulbecco's modified Eagle's medium containing 10% fetal calf serum, and 1% ethanol that had been pre-equilibrated at 45°C, and flasks were incubated at 45°C for 30 min. Medium was removed and replaced with medium pre-equilibrated at 37°C containing 10% fetal calf serum and 1% ethanol and MNNG at different concentrations, and cells were returned to the 37°C incubator for different periods of time. Medium was decanted and flasks were rinsed three times with cold PBS, and 20% TCA was added. Acid insoluble material was obtained by scraping the flasks and centrifugation of the acid insoluble material in the cold at 1000xg for 10 min.

Purification of Poly(ADP-ribose) by Affinity Chromatography

The TCA pellets were routinely dissolved in 2.0 ml 0.2 N NaOH, 20 mM EDTA by incubation at 60°C for 2 hours. When the material was labeled with [^{3}H] Ade, the incubation was made at 37°C rather than to 60°C to prevent chemical loss of radiolabel by tritium exchange reactions. The solution was

then diluted five-fold with 0.25 M NH_4OAc buffer, pH 9.0, 6 M Gu.HCl, and the pH was adjusted to 9.0 by the addition of approximately 100 µl of concentrated HCl. An Econo-Column containing 0.5 ml of DHB-Bio Rex was equilibrated with 10 ml of 0.25 M NH_4OAc buffer, pH 9.0, containing 6.0 M Gu.HCl. The sample was applied to the boronate resin followed by a 10 ml wash of application buffer and two 10 ml washes with 1 M NH_4HCO_3 buffer, pH 9.0, and it was eluted with 5.0 ml of water pre-equilibrated to 37°C. The yield of radiolabeled poly(ADP-ribose) was routinely above 80%. The material was lyophilized, dissolved in water, lyophilized again and redissolved in 50 to 200 µl of water and kept frozen at -20°C until used.

Polyacrylamide Gel Electrophoresis of Poly(ADP-ribose)

Polyacrylamide gels of 20% acrylamide and bis-acrylamide, in a ratio of 19:1 containing 100 mM Tris-borate buffer, pH 8.3, 2 mM EDTA ammonium persulfate, and 3.4 mM TEMED were used. Pre-electrophoresis was carried out for 1 to 2 hours at 400 volts using 50 mM Tris-borate, pH 8.3, containing 1 mM EDTA as the electrode buffer. Samples of 10 to 20 µl containing 20,000 to 40,000 cpm of [^{32}P] poly(ADP-ribose) and 50% urea, 25 mM NaCl, 4 mM EDTA pH 7.5, 0.02% XC and 0.02% BPB (Electrophoresis loading buffer) were applied to the gels, and electrophoresis was carried out at 400 or

800 volts until the BPB migrated 9 cm from the origin in 20 cm long gels, and 19 cm from the origin of 40 cm gels. At this point, electrophoresis was stopped and the gel was autoradiographed at -80°C with the aid of an intensifying screen.

Molecular Sieve Chromatography of Poly(ADP-ribose)

Purified polymers of ADP-ribose were fractionated by size exclusion chromatography in a Beckman model 330 liquid chromatograph using a Bio-Sil TSK-125 column (300 mm x 7.5 x mm I.D.) that was preceded by a guard column (75 mm x 7.5 mm I.D.) containing the same material. The injector was equipped with a 1.0 ml sample loop. An Instrumentation Specialties Company UA-5 ultraviolet absorbance monitor was used to monitor absorbance at 254 nm. The absorbance monitor was equipped with a Type 6 optical unit, 19 µl flow cells, and a 254 nm bandpass filter. The running solvent used was 0.1 M sodium phosphate buffer, pH 6.8 at a flow rate of 1 ml/min. Samples were routinely injected in 1 ml and fractions of 500 1 were collected. Small aliquots were removed from each fraction for determination of the amount of radioactivity.

Purification of Snake Venom Phosphodiesterase

A Blue-Sepharose column (0.7 x 7 cm) was used to purify SVPD according to the procedure described by Hayaishi and co-workers (135), with a slight modification. The chromatography was run at room temperature and the flow rate was 12 ml/hour. The column was washed with 50 ml of 3 M KCl and then equilibrated with 50 ml of 10 mM Tris-HCl, 50 mM NaCl, 5 mM potassium phosphate, pH 7.5. One hundred units (3 mg) of enzyme was dissolved in 1.5 ml of 10 mM Tris-HCl, 50 mM NaCl, 5 mM potassium phosphate, pH 7.5 and loaded on the column followed by a 20 ml wash with the same buffer solution. The column was then washed with 20 ml of 10 mM Tris-HCl, 50 mM NaCl, 30 mM potassium phosphate, pH 7.5 and the enzyme activity was eluted with 10 ml of 10 mM Tris-HCl, 50 mM NaCl, 100 mM potassium phosphate, pH 7.5. Most of the original phosphodiesterase activity (77%) was recovered free of 5' nucleotidase activity. The purified enzyme was then concentrated and dialyzed against 10 mM Tris-HCl, 50 mM NaCl, 30 mM potassium phosphate, pH 7.5, containing 20% glycerol. The enzyme was stored in the same buffer at -20°C until used at a final concentration of 77 units/ml.

Enzymatic Digestion of Poly(ADP-ribose) To Nucleotides

Fractions of 500 µl containing polymers of ADP-ribose size collected by molecular sieve chromatography were diluted to 1.0 ml with water. Samples were adjusted to pH 8.0, and a final concentration of 10 mM $MgCl_2$ and subjected to treatment with 0.2 units of SVPD for 2 hours at 37°C.

Strong Anion Exchange High Performance Liquid Chromatography (SAX-HPLC)

Nucleotides obtained following SVPD digestion of poly(ADP-ribose) were separated on the same chromatographic apparatus described above using a Whatman PARTISIL-10 SAX column (250 mm x 4.6 mm I.D.), preceeded by a guard column (50 mm x 1.5 mm I.D.) containing the same material. KCl was added to a final concentration of 50 mM and samples were injected to a volume of 1.0 ml. The column was eluted isocratically with 125 mM potassium phosphate, pH 4.7, containing 0.5 M KCl at room temperature at a flow rate of 1.0 ml/min. Radioactivity was monitored by collecting 1.0 ml fractions for scintillation counting and the relative amount of radiolabel in each nucleotide was used to calculate the average polymer size as well as the average

number of points of branching per molecule as described in Results.

Alkaline Digestion of Purified Poly(ADP-ribose)

Poly(ADP-ribose) was treated with either 0.1 N or 0.2 N NaOH in the presence of either 20 mM EDTA or 10 mM $MgCl_2$ for 3 hours at 60°C in a final volume of 10 µl. The alkaline treatment was terminated by neutralization to pH 7.5 with 6 N HCl. The samples were diluted three fold with loading buffer for electrophoresis on 20% polyacrylamide gels.

Preparation of the Mixture of the Enzymes SVPD and BAP

A mixture of 1000 units of BAP and 100 units of SVPD was incubated for 2 hours at 37°C in a final volume of 10 ml of 10 mM MOPS buffer, pH 7.4 contaiing 50 mM $MgCl_2$ to digest RNA and DNA which were present as contaminants in the commercial BAP preparations. This solution was then dialyzed in the cold against 1 l of 10 mM MOPS buffer, pH 7.4, containing 50 mM $MgCl_2$ for 24 hours with buffer changes at 8 hour intervals. The dialysate was diluted to 40 ml with the same buffer and stored frozen at -20°C in 1 ml aliquots until used.

Enzyme Digestion of Poly(ADP-ribose) To Nucleosides

Purified polymers of ADP-ribose were incubated for 3 hours at 37°C with 100 µl of a mixture of SVPD and BAP in a final volume of 5 ml containing 10 mM $MgCl_2$ and 25 mM NH_4OAc buffer, pH 9.0.

Formation of $1,N^6$-etheno Nucleosides

The nucleosides adenosine, ribosyladenosine and diribosyladenosine obtained following polymer digestion with SVPD and BAP were incubated for 4 hours at 60°C in a final volume of 5.5 ml in the presence of 0.25 M NH_4OAc buffer, pH 4.5 and 100 mM chloroacetaldehyde. This resulted in the quantitative conversion of the nucleosides to their respective $1,N^6$-etheno-derivatives (85).

Preparation of $1,N^6$-etheno Nucleosides for Fluorescent Analysis

Solutions containing the $1,N^6$-etheno nucleosides were diluted to 10 ml containing a final concentration of 0.25 M NH_4OAc buffer, and the pH was adjusted to 9.0 by the addition of concentrated NH_4OH (approximately 200-250 µl). The solution was centrifuged at low speed to remove any insoluble material. The sample was then applied to a 0.5 ml

column of Matrex gel PBA-60 previously equilibrated with 5 ml of 0.25 M NH_4OAc buffer, pH 4.5 followed by 10 ml of 0.25 M NH_4OAc buffer, pH 9.0. Following application of the sample the column was washed with 10 ml of the application buffer and the fluorescent nucleoside derivatives were eluted with 4.0 ml of 200 mM sodium citrate buffer, pH 4.0.

Fluorescent Analysis of 1,N^6-etheno Nucleosides by Reversed-phase HPLC

The fluorescent nucleosides derived from poly(ADP-ribose) were separated on a Beckman-Altex ultrashpere-ODS reversed-phase column (250 mm x 4.6 mm I.D.). The chromatographic apparatus was the same as describe above, except that it was equipped with an injector loop of 2.0 ml. All samples were injected in a total volume of 2.0 ml in 200 mM sodium citrate buffer, pH 4.5. The column was eluted isocratically at a flow rate of 1.0 ml/min at room temperature with a mixture of 7 mM ammonium formate buffer, pH 5.8 and 100% methanol. The relative ammounts of buffer and methanol (v/v) were varied in the range of 6 to 10% methanol according to the age of the column, so that retention times of the etheno nucleosides remained constant. Fluorescence was monitored was with a Varian Fluorichrom filter fluorimeter equipped with a deuterium light source, a 220 I excitation filter and a 370 nm cut-off emission filter.

CHAPTER IV

RESULTS

Development of a Method to Estimate the Size and Complexity of Poly(ADP-ribose)

Purification of poly(ADP-ribose) on Dihydroxyboronyl Bio-Rex by affinity chromatography.- The structure of poly(ADP-ribose) is very similar to other macromolecules such as RNA or DNA, which occur in cells in far greater amounts. Thus, it is necessary to purify polymers of ADP-ribose from these interfering materials. It has previously been shown that immobilized boronates can effectively be utilized for this purpose. However, the amount of the boronate ligand incorporated onto the insoluble support needs to be carefully controlled (7,9). Dihydroxyboronyl Bio-Rex, a boronate resin synthesized in this laboratory was found to be very effective for the purification of poly(ADP-ribose). This resin requires that the molecule to be retained contain at least two sets of vicinal hydroxyl groups. Thus, poly(ADP-ribose) is retained on the resin while most other nucleic acids are not. The advantages of using this resin, over other immobilized boronates, to study

pyridine nucleotide metabolism and its use to measure total levels of monomeric and polymeric ADP-ribose *in vivo* have already been published (9).

The treatment of acid insoluble material with SVPD may generate AMP from protein bound mono(ADP-ribose) and from RNA as well as from the non-reducing termini of polymers of ADP-ribose Therefore, in order to accurately determine the average polymer size, it was important to make certain that AMP was derived from only poly(ADP-ribose). To avoid AMP contamination from RNA or protein bound mono(ADP-ribose), the acid insoluble material was subjected to alkaline treatment in the presence of EDTA. Under these conditions the anhydride linkages of poly(ADP-ribose) are stable (see below), while the phosphoanhydride bond of mono(ADP-ribose) and and the phosphodiester linkages of RNA are not. Table II shows that alkaline treatment in the presence of EDTA prior to boronate chromatography totally eliminated contamination from monomeric (ADP-ribose) residues, since monomers were converted to AMP and thus not retained by the boronate resin. This treatment should also have resulted in the hydrolysis of the covalent linkages between poly(ADP-ribose) and protein since most of them are very labile under mildly alkaline conditions. The known covalent attachment of the polymer to protein involves an ester linkage which is hydrolyzed at pH values above 7.0.

TABLE II

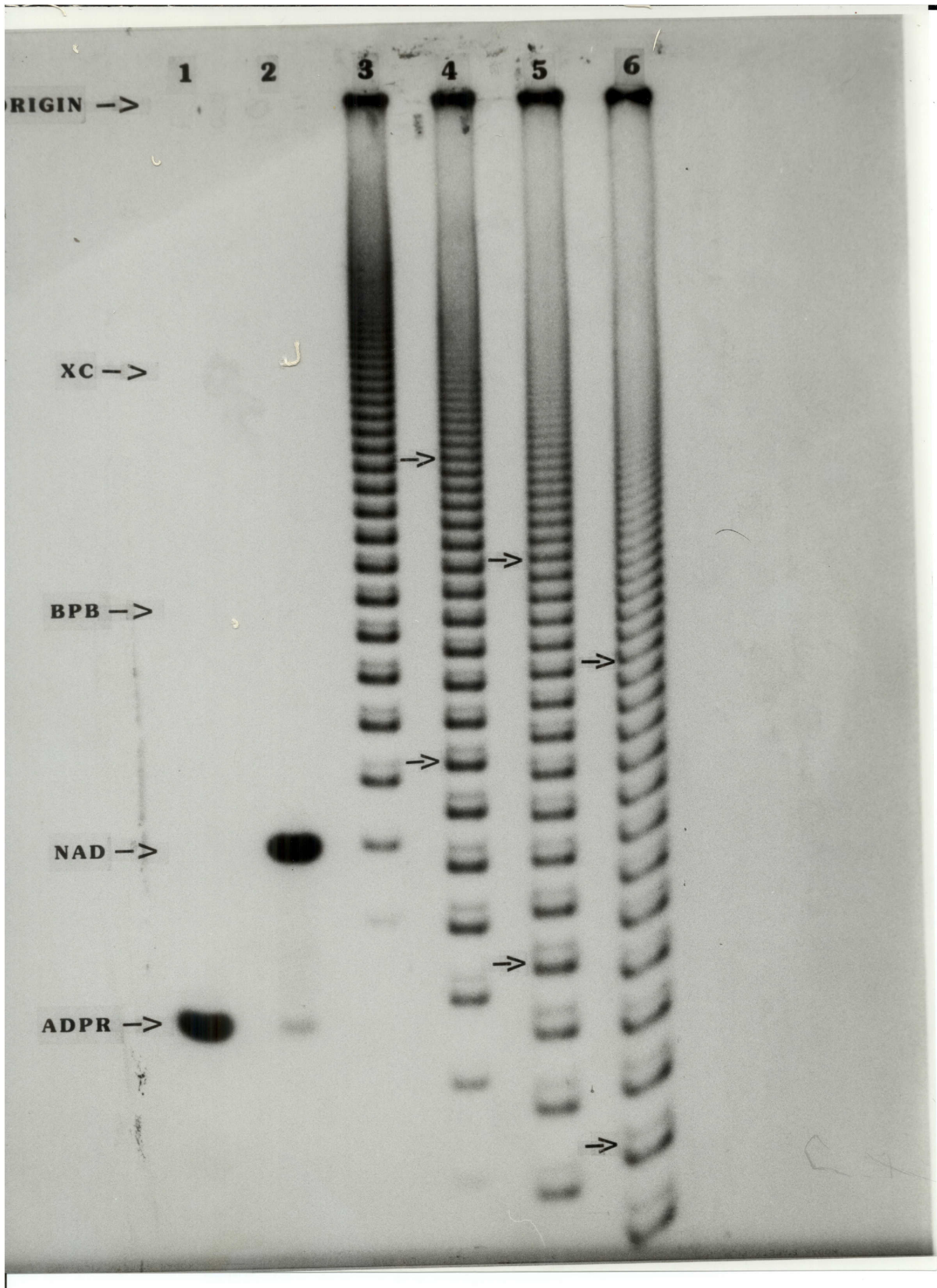
Binding Of Protein-Bound Monomeric And Polymeric Residues Of ADP-ribose to DHB-Bio Rex Following Alkaline Treatment

Sample	Radioactivity(cpm)		% Bound
	Total	Bound	
Poly(ADP-ribose)	6958	6761	97.1
Mono(ADP-ribose)	7901	42	0.5

Purified poly(ADP-ribose) and mono(ADP-ribosylated) histone H1 were incubated with 1.0 M KOH, 50 mM EDTA at 60°C for 1 hour. The pH was adjusted to 9.0 by the dropwise addition of concentrated HCl and the samples were chromatographed on DHB-Bio Rex as indicated in methods.

Fractionation of purified poly(ADP-ribose) according to size.- Initial studies for the characterization of poly(ADP-ribose) were designed to evaluate methods for the separation of polymers according to size. The polymer size distribution was first analyzed by electrophoresis on 20% polyacrylamide gels using [^{32}P] labeled polymers generated in nucleotide permeable cells. Figure 7 shows the electrophoretic separation of polymers of ADP-ribose. Lanes 1 and 2 show the relative migration of ADP-ribose and NAD^+. Samples in lanes 1, 2 and 3 were loaded on the gel at the same time, while samples in lanes 4, 5 and 6 represent polymers of ADP-ribose subjected to electrophoresis for longer periods of time. It is important to note that the fastest migrating band corresponds to a dimer of ADP-ribose since this preparation had been treated with alkali prior to electrophoresis, which eliminated ADP-ribose. The resolving power of this method was very good as polymers differing in size by a single residue were separated. It was observed that the dyes BPB and XC co-migrated with oligo (ADP-ribose) of an average size of approximately 8 and 20 residues, respectively. This agreed with previous work of Tanaka et al. (159). The positive charge in the pyridine ring od the oxidized NAD^+ retards its migration on polyacrylamide gels, such that NAD^+ migrates at the position of a trimer of ADP-ribose. It was noted that when 40 cm gels were used, polymers of different sizes were resolved as ladders of

Figure 7. Polyacrylamide gel electrophoresis of purified poly(ADP-ribose). Lanes 1 and 2 show the relative migration of mono(ADP-ribose) and NAD^+ respectively. Lanes 3 to 6 show samples of poly(ADP-ribose) subjected to electrophoresis for different lengths of time at 400 volts, (3,4,5 and 6 hours respectively). Abbreviations: BPB, bromophenol blue. XC, xylene cyanol. The arrows in lanes 4, 5 and 6 show the relative migrations of XC and BPB, respectively.


1
2
3
4
5
6
RIGIN ->
XC ->
BPB ->
NAD ->
ADPR ->

souble bands. experimental material was sometimes isolated under conditions that avoided treatment with strong alkali. Under these conditions triple bands were observed as shown in figure 8. It was possible that the multiple bands were due to differences in migration between linear and branched polymers. Thus, the source of the multiple banding was studied. Hilz and co-workers (67) have shown that the phosphoanhydride linkage of the reducing terminal ADP-ribose residue of a free polymer is unstable under alkaline conditions. This is due to an intramolecular interaction between the Ade distal rital phosphate which results in the generation of a cyclic intermediate. Therefore, it was possible that under the mild alkaline conditions used for the purification of poly(ADP-ribose), polymers would be heterogeneous at the reducing end as shown in figure 9. The polymer free of protein may be converted to one carrying only a terminal phosphate following the loss of the ribose-phosphate portion of the reducing end. In addition, this polymer species may lose the terminal phosphate either enzymatically or chemically, yielding hydroxylated species of the polymer. Figure 8 shows that treatment of the preparations containing multiple banding with 0.2 N NaOH in the presence of EDTA resulted in the disappearance of one band of each triplet. Thus, the bands that disappear can be ascribed to polymers with an intact ADP-ribose residue at the reducing end. Figure 8 also shows that one of the two

Figure 8. Characterization of poly(ADP-ribose) by electrophoresis on polyacrylamide gels. Lane 1 shows the electrophoretic profile of poly(ADP-ribose) isolated under mild alkaline conditions (control). Lane 2 shows poly(ADP-ribose) treated with 10 units/ml of BAP at 37°C for 1 hour in 25 mM NH_4OAc, pH 9.0. Lane 2' shows poly(ADP-ribose) treated with 0.2 N NaOH, 20mM EDTA at 60°C for 3 hours. Lane 3' shows poly(ADP-ribose) treated with 0.2 N NaOH, 20 mM EDTA at 60°C for 3 hours followed by incubation with 10 units/ml of BAP at 37°C for 1 hour in the presence of 25 mM NH_4OAc, pH 9.0. Abbreviations: O, origin of application. XC, xylene cyanol. BPB, bromophenol blue. The ribosylated and phosphorylated forms of poly(ADP-ribose) are abbreviated by an R and a P to the left of lane 1. The migration of the hydroxylated forms of poly(ADP-ribose) is abbreviated by OH to the right of lane 3'. Polymer size is indicated by an arabic number to either the left or right of the figure. The arrows indicate the postulated effects on the migration of at least one of the three bands observed for each polymer size following enzyme and/or chemical treatment. The dotted lines show equivalent polymer species between different lanes.

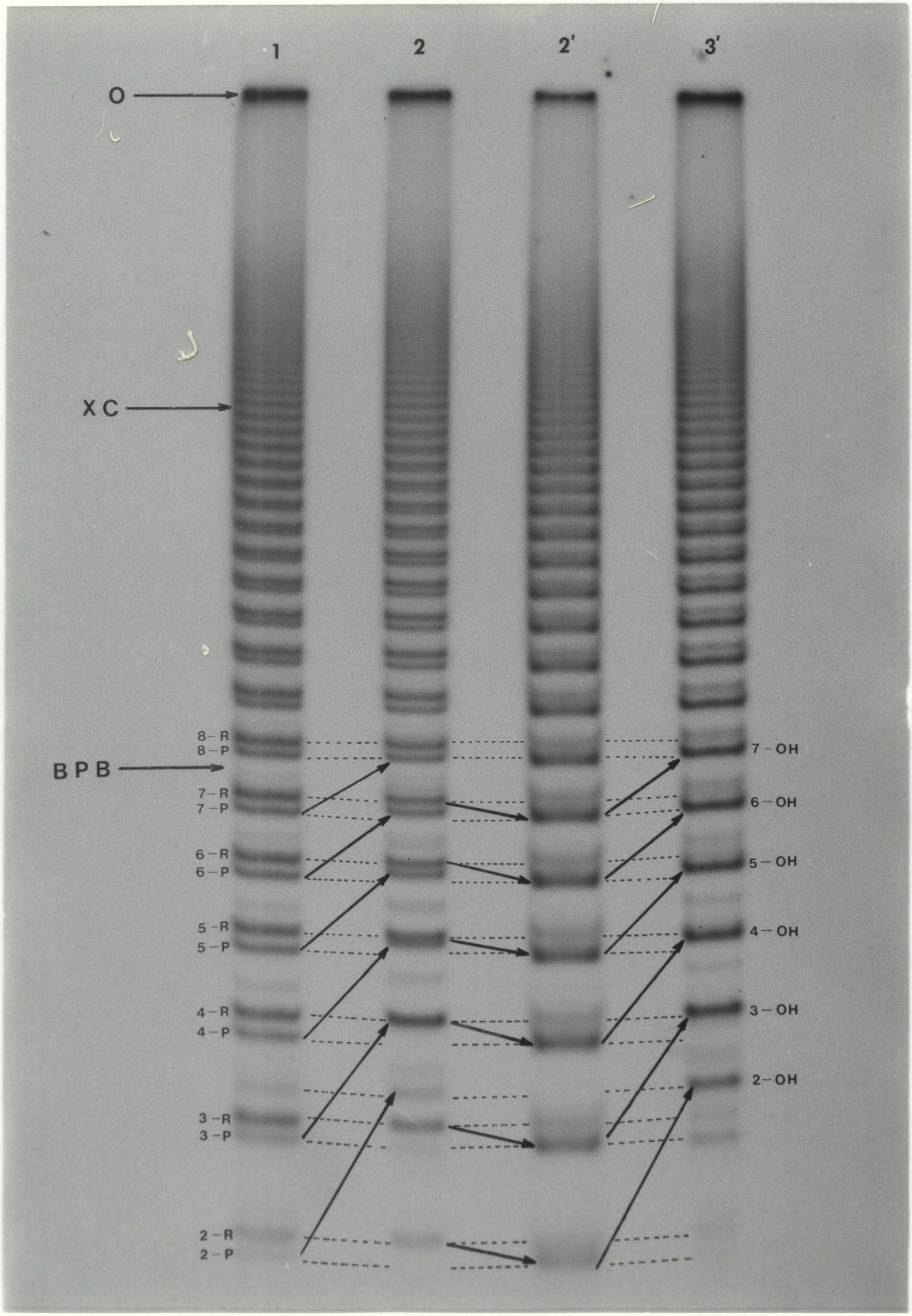
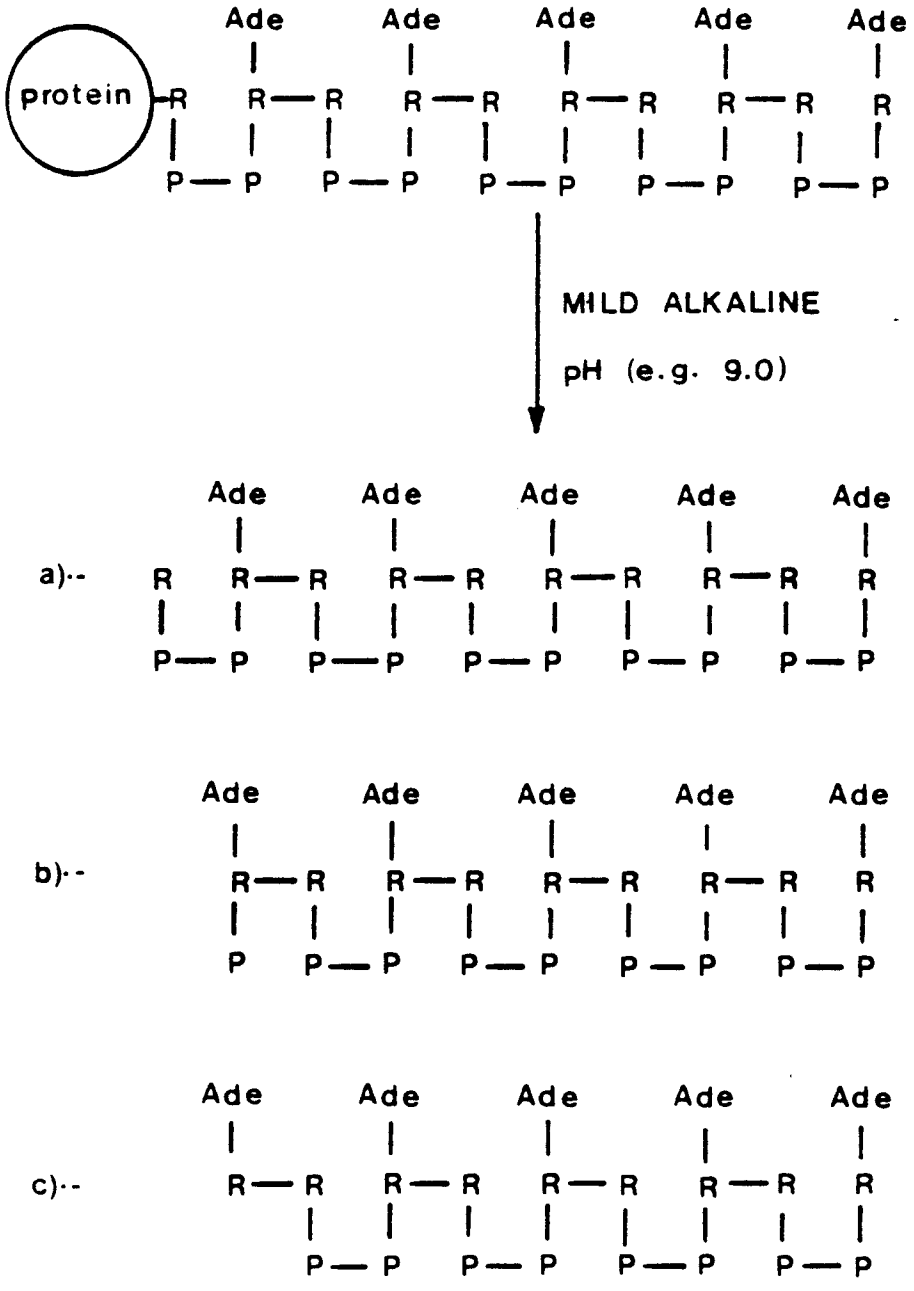

1
2
2'
3'
O
XC
BPB
8-R
8-P
7-R
7-P
6-R
6-P
5-R
5-P
4-R
4-P
3-R
3-P
2-R
2-P
7-OH
6-OH
5-OH
4-OH
3-OH
2-OH

Figure 9. Release of poly(ADP-ribose) from protein under mild alkaline conditions. Abbreviated structure of the three classes of polymer generated upon release from protein. A linear polymer of ADP-ribose is used, only for purposes of simplicity. a).- Pentamer of ADP-ribose with an intact reducing end. b).- Pentamer of ADP-ribose with a terminal phosphate. c).- Pentamer of ADP-ribose with a 5' hydroxyl group.


Ade
protein
R
P
MILD ALKALINE
pH (e.g. 9.0)
a)··
b)··
c)··

polymer species resistent to alkaline conditions was sensitive to BAP. Thus, these species may be attributed to phosphorylated forms of the polymer. The polymer species obtained following BAP treatment (lanes 2 and 3') should correspond to the hydroxylated forms. This interpretation is further supported by the fact that 5'-hydroxylated oligonucleotides migrate more slowly on gel electrophoresis than the respective phosphorylated analogues (13). In total, the data shown in figure 8 are consistent with the interpretation that the multiple banding observed on 40 cm polyacrylamide gels can be accounted for by heterogeneity at the reducing end of the polymers. Thus, they are not likely to be due to differences of migration between linear and branched polymers of ADP-ribose.

It is clear that polyacrylamide gel electrophoresis is a powerful analytical tool for fractionation of polymers of ADP-ribose according to size. However, determination of polymer size and complexity in vivo also requires sensitive detection and high recovery. With regard to detection, two different radiolabeling approaches were evaluated for the synthesis of radiolabeled poly(ADP-ribose) in intact cells. One utilized [^{3}H] Ade and a second used [^{32}P] inorganic phosphate to label the NAD^{+} pool in vivo. Therefore, the sensitivity of detection by autoradiography of [^{3}H] labeled and [^{32}P] labeled poly(ADP-ribose) synthesized in nucleotide permeable cells, was compared. The results are shown in

Figure 10. Sensitivity of detection by autoradiogrphy of [^{3}H] labeled and [^{32}P] labeled poly(ADP-ribose) synthesized in nucleotide permeable cells. The conditions of electrophoresis were as described in methods except that the gel contained 8 M urea. Lanes A, B, C and D on the left side show 800,000; 400,000; 200,000 and 100,000 cpm of [^{3}H] labeled poly(ADP-ribose), respectively. Lanes A and B on the right side show 500 and 1,000 cpm of [^{32}P] labeled poly(ADP-ribose), respectively. Abbreviations: xc, xylene cyanol and bpb, bromophenol blue.

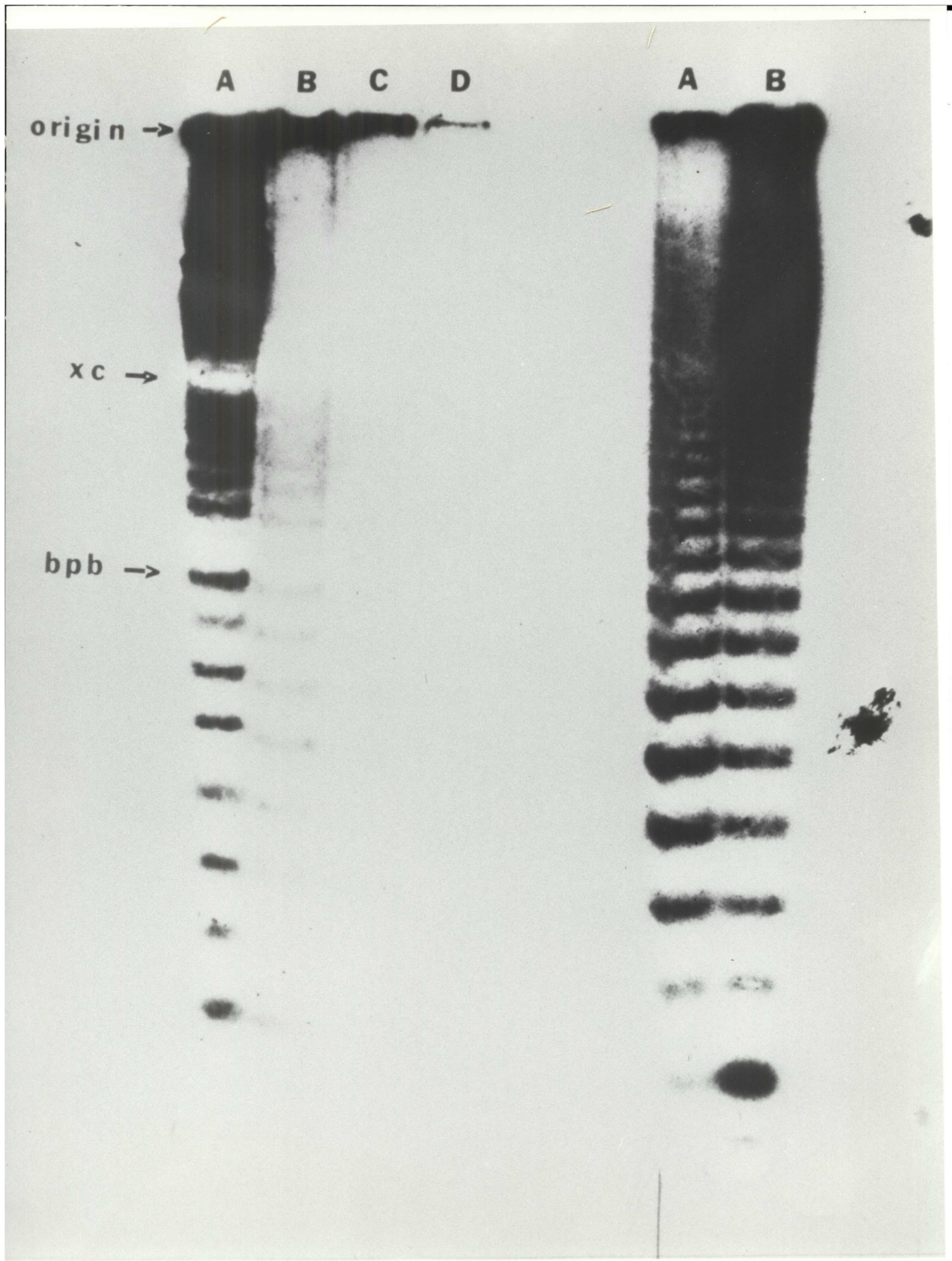

A
B
C
D
origin
xc
bpb
A
B

figure 10. Approximately 800,000 cpm of [^{3}H] labeled poly(ADP-ribose) were detected following two weeks of autoradiography. In contrast, 500 cpm of [^{32}P] labeled poly(ADP-ribose) were readily detected. These results were obtained with the aid of an autoradiography intensifying screen at -80°C, following treatment of the gel with autoradiographic enhancer. Therefore, detection of [^{3}H] labeled poly(ADP-ribose) on polyacrylamide gels was not nearly as efficient. In addition, in order to analyze polymer complexity, one must be able to recover the material following fractionation with high efficiency. The yield of radiolabeled poly(ADP-ribose) following extraction from the polyacrylamide gels was low. Recoveries never exceeded 70%. In addition, a considerable amount of material (30%) that did not migrate into the gels was consistently observed (figures 7 and 8). In an attempt to improve recoveries, gels were polymerized using the hydrolyzable bis-acrylamide analogue (N,N',bis-acrylcystamine) as the cross-linker. However, the fractionation of poly(ADP-ribose) according to size on this gels was not as efficient. Therefore, it was concluded that polyacrylamide gel electrophoresis was not acceptable for polymer characterization, especially for polymers synthesized <u>in vivo</u>. Nevertheless, this technique was a valuable analytical tool for validation of other fractionation methods.

Molecular sieve chromatography was evaluated for its utility in polymer fractionation. Figure 11 shows the separation of poly (A) (greater than 100 nucleotides), $tRNA^{Tyr}$ (76 nucleotides), a decamer of adenylic acid [$(Ap)_9A$], and AMP on a Bio-Sil TSK-125 column. Since an efficient resolution of these polynucleotides was observed, this method was used to fractionate poly(ADP-ribose). Figure 12 shows the absorbance and radioactivity profiles obtained when [^{32}P] labeled poly(ADP-ribose) was analyzed by molecular sieve chromatography. The distribution of radiolabel was identical to the absorbance profile and it spanned the entire range from the void volume to the included volume. Note that very little radiolabel was observed in fraction number 27, the position at which AMP, ADP-ribose and NAD^+ eluted. It is important to note that the recovery of radiolabeled material following molecular sieve chromatography was routinely above 95%. Therefore, this methodology satisfied this criterion for polymer characterization. In order to examine the resolution obtained by molecular sieve chromatography, each of the fractions collected was subjected to electrophoresis on 20% polyacrylamide gels. Figure 13 shows that the fractions collected following molecular sieve chromatography contained polymers of ADP-ribose that behaved electrophoretically different. Fractions 16 to 25 contained a narrow population of poly(ADP-ribose) molecules of different average size as

Figure 11. Molecular sieve chromatography of polynucleotide standards using a TSK-125 column. The arrows indicte void volume and included volume, respectively. Peaks 1, 2, 3 and 4 correspond to poly (A), [^{32}P] tRNA^{Tyr}, $(Ap)_9A$, and AMP, respectively. (●——●) Radioactivity, (———) absorbance.

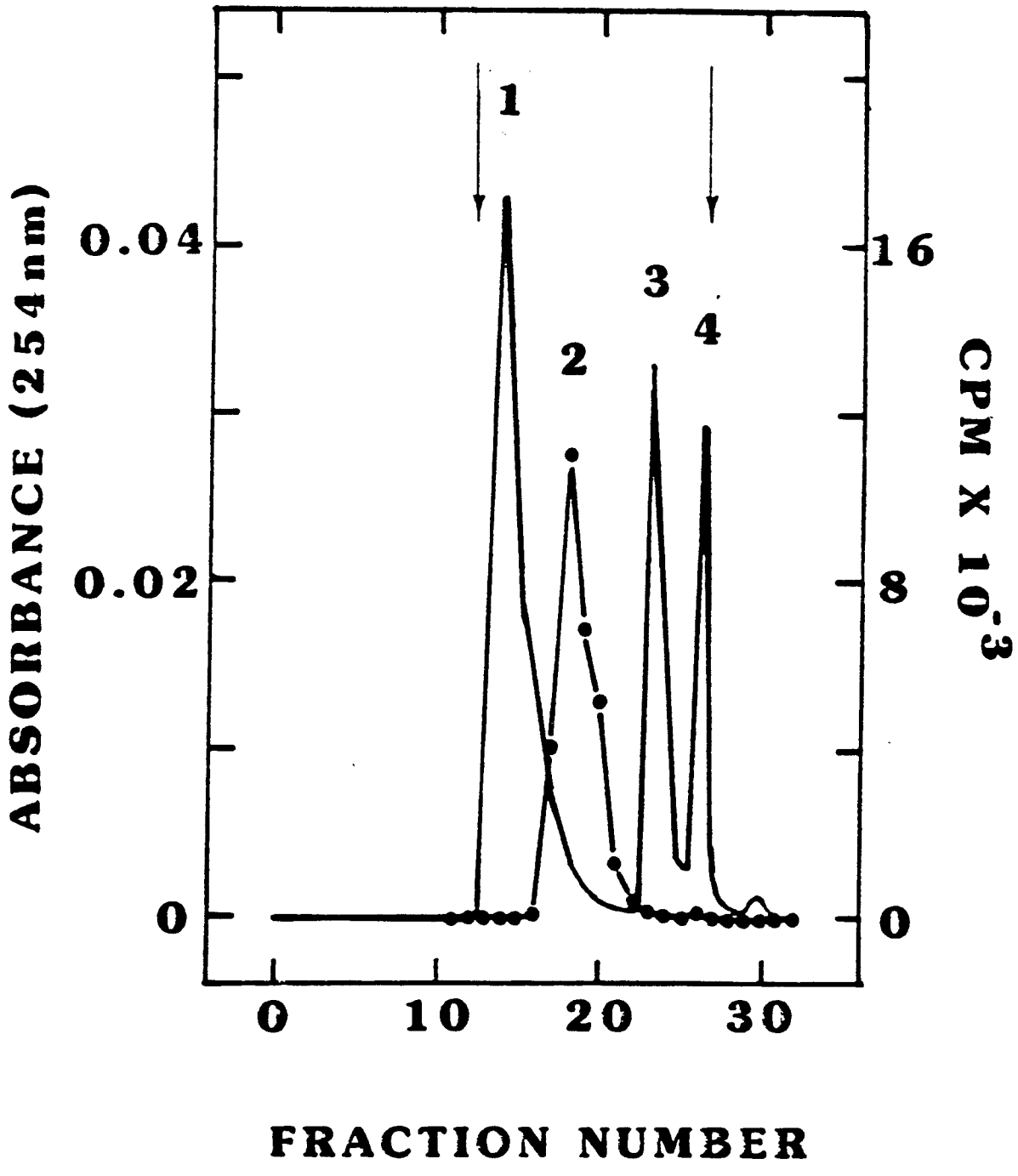

ABSORBANCE (254nm)
0.04
0.02
0
1
2
3
4
16
8
0
CPM X 10.3
0
10
20
30
FRACTION NUMBER

Figure 12. Molecular sieve chromatography of [^{32}P] poly(ADP-ribose) made in nucleotide permeable cells. Vo indicates void volume. The retention times of polymers of ADP-ribose of known size are also indicated. (——) absorbance at 254 nm. (●——●) radioactivity.

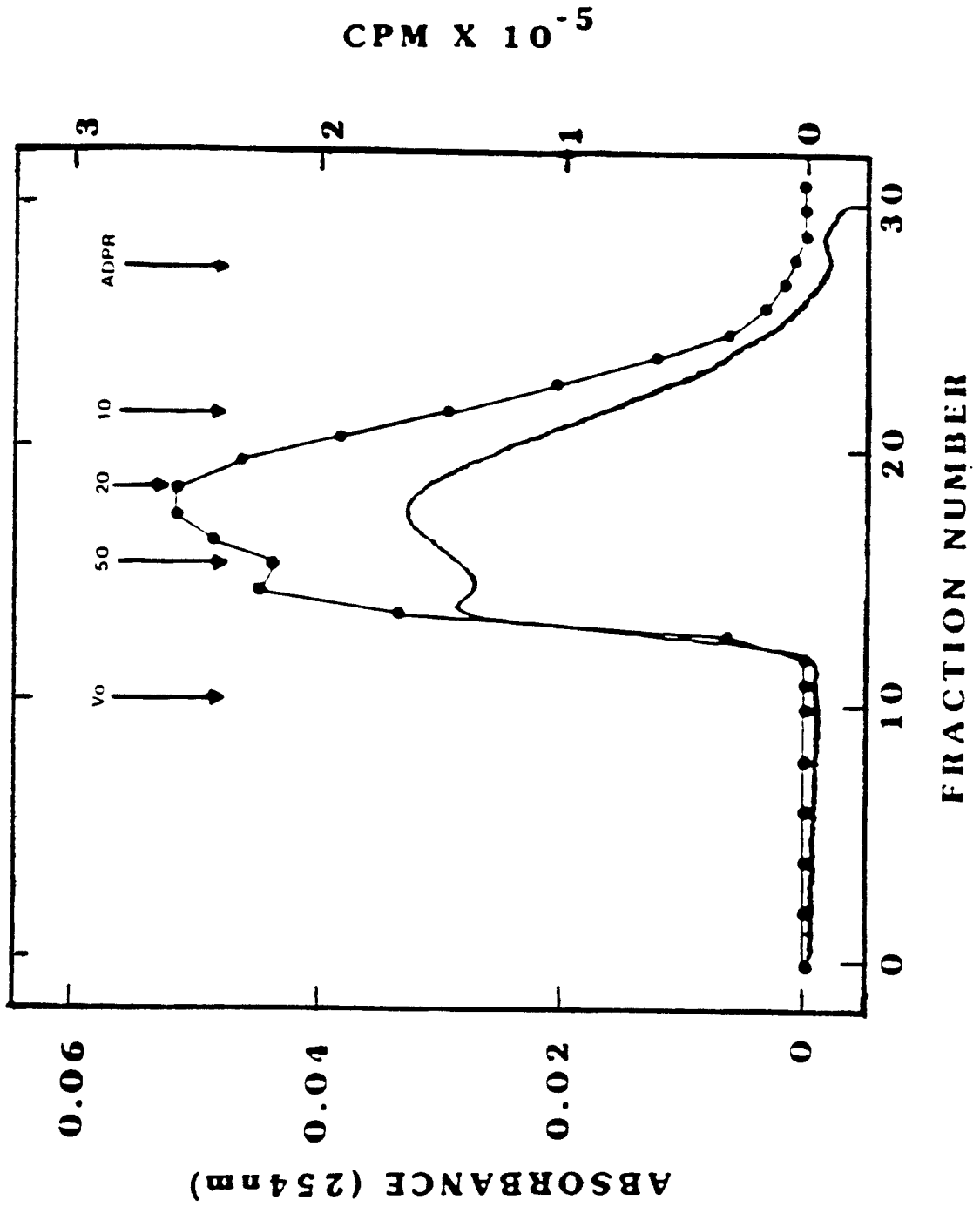
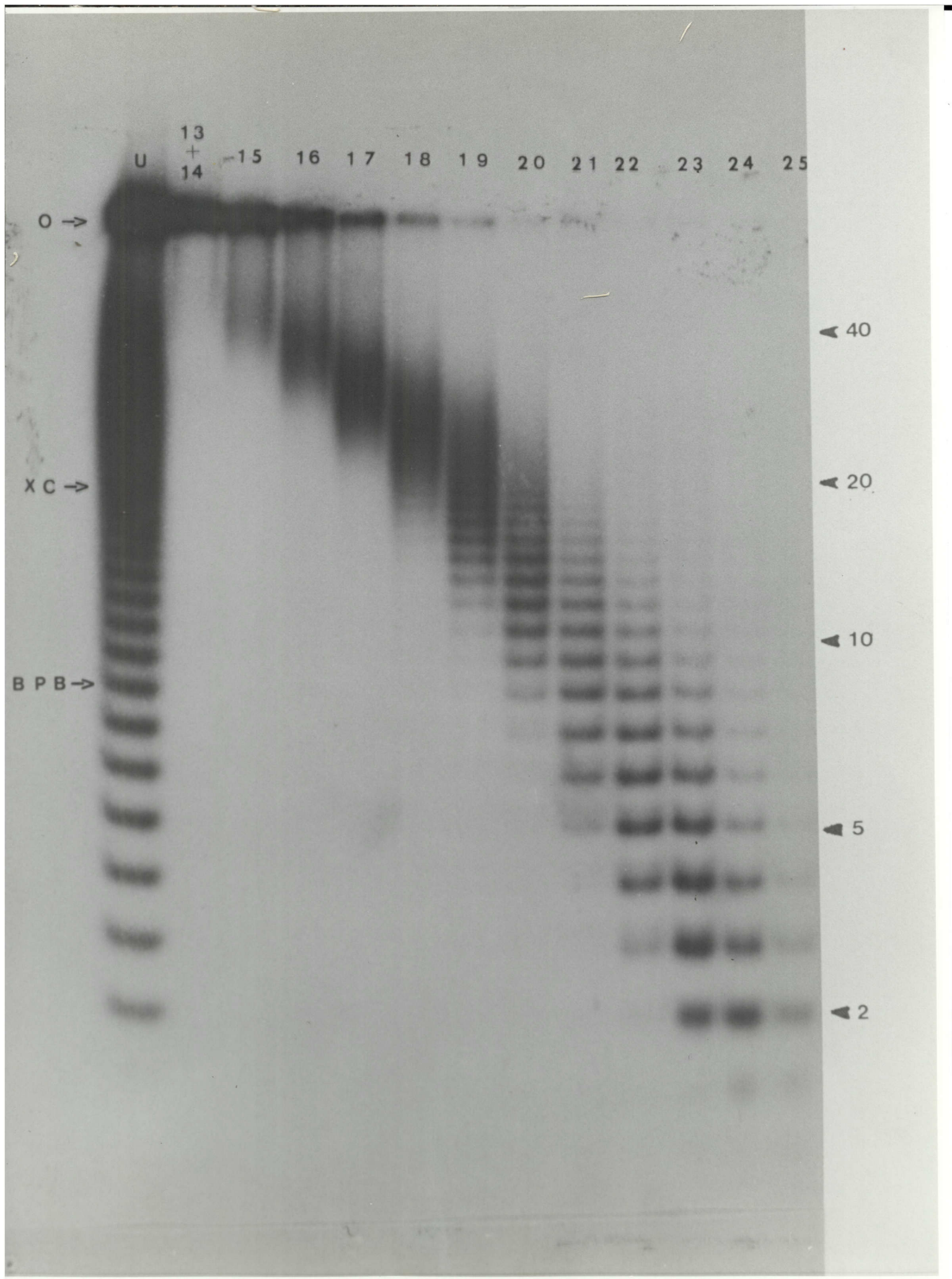

CPM X 10-5
3
2
1
0
ADPR
10
20
50
Vo
0.06
0.04
0.02
0
ABSORBANCE (254nm)
0
10
20
30
FRACTION NUMBER

Figure 13. Polyacrylamide gel electrophoresis of poly(ADP-ribose) fractions collected following molecular sieve chromatography. The conditions of electrophoresis were as described in methods except that the gel also contained 8 M urea. Abbreviations: U, unfractionated polymer (control). BPB, bromophenol blue. XC, xylene cyanol. The relative migrations of polymers of ADP-ribose of known size are also indicated.

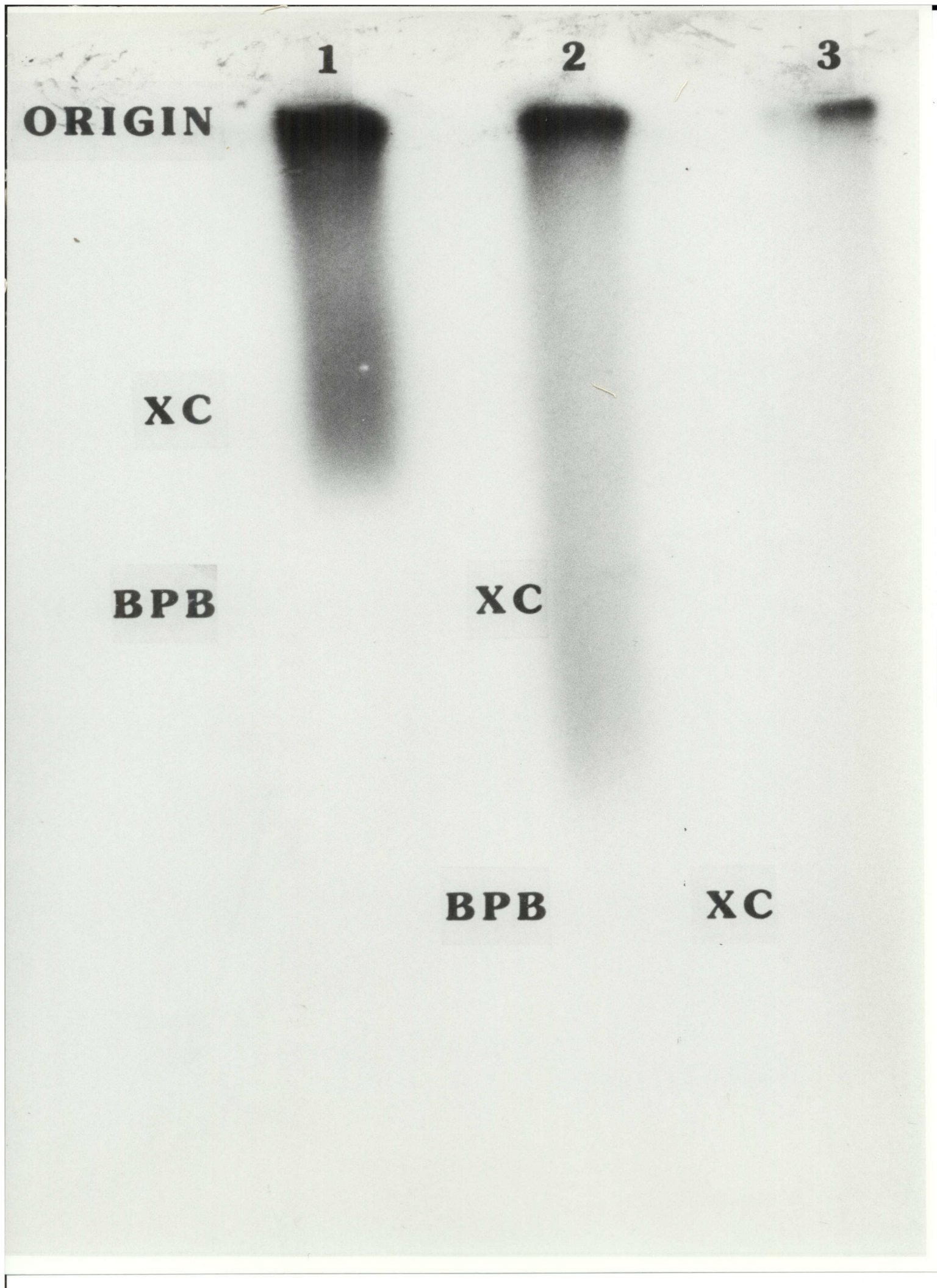

U
13 + 14
15
16
17
18
19
20
21
22
23
24
25
O →
X C →
B P B →
40
20
10
5
2

judged by relative migration. Again it was observed that about 30% of the total radiolabeled material did not migrate on the gels.

Figure 14 demonstrates that these polymers did not effectively migrate on 4% polyacrylamide gels either. Lanes 1, 2 and 3 show the [^{32}P] poly(ADP-ribose) subjected to polyacrylamide gel electrophoresis for different lengths of time. Approximately 75% of the radiolabeled polymers remained at the origin of the gels upon electrophoresis. It is interesting to note that the small amount of material that migrated into the gel did not resolve into discrete bands. In order to evaluate the complexity of poly(ADP-ribose), it was necessary to determine whether the preparations contained polymers free of covalent attachment to other macromolecules, i.e., DNA, RNA or protein. Thus, the preparations of poly(ADP-ribose) were further characterized to determine if they contained other chromatin components covalently bound.

Characterization of poly(ADP-ribose).- To determine if poly(ADP-ribose) preparations were free of covalent attachment to protein, polymers were analyzed by either polyacrylamide gel electrophoresis or molecular sieve chromatography following treatment with proteases or nucleases. Most of the poly(ADPribose)-protein bonds are very sensitive to alkaline conditions. However, it has been suggested that the chemical stability of these linkages is

Figure 14. Profile of complex poly(ADP-ribose) upon electrophoresis in 4% polyacrylamide gels. The same material was electrophoresed for 1, 2 and 4 hours on lanes 1, 2 and 3. Abbreviations: XC, xylene cyanol. BPB, bromophenol blue.


1
2
3
ORIGIN
XC
BPB
XC
BPB
XC

heterogeneous because of the resistance of a significant fraction of the conjugates (20 to 30%) to dilute alkali or neutral hydroxylamine (67). Therefore, it was possible that the material that did not migrate into the gels corresponded to polymers that were still attached to protein via an alkali stable linkage. Thus, fractions 13 to 15, containing complex polymers were pooled and subjected to various enzyme treatments, followed by rechromatography on a TSK-125 column. Figure 15 shows that the untreated material still migrated near the void volume upon rechromatography. In addition, the migration was unaffected by treatment with either proteinase K or pronase E. Incubations containing a mixture of thyroglobulin, IgG, myoglobin and ovalbumin demonstrated that these proteases were active under the conditions employed (figure 16). Unfractionated poly(ADP-ribose) was also subjected to polyacrylamide gel electrophoresis following treatments with proteinase k and trypsin. The electrophoretic profile was not affected as demonstrated in figure 17.

Figure 15 also shows that incubation with SVPD generated radiolabeled products that eluted at the included volume. Figure 18 demonstrates that the SVPD digestion products corresponded to PRAMP and AMP as judged by polyacrylamide gel electrophoresis. The migration of PRAMP on polyacrylamide gels is faster than that of AMP because it has two additional negative charges.

Figure 15. Molecular sieve chromatography of complex polymers of ADP-ribose following different enzyme treatments. The arrows indicate the void volume and the included volume respectively. The chromatographic conditions used were the same as indicated in figure legend 13. (■—■) untreated material. (▲—▲) treatment with 1 unit/ml of pronase E at 37°C for 3 hours at pH 7.4. (●—●), treatment with 1 unit/ml of proteinase k at 37°C for 3 hours at pH 7.4. (○—○) treatment with 0.2 units/ml of SVPD at 37°C for 3 hours at pH 9.0 in the presence of 25 mM NH_4OAc pH 9.0.

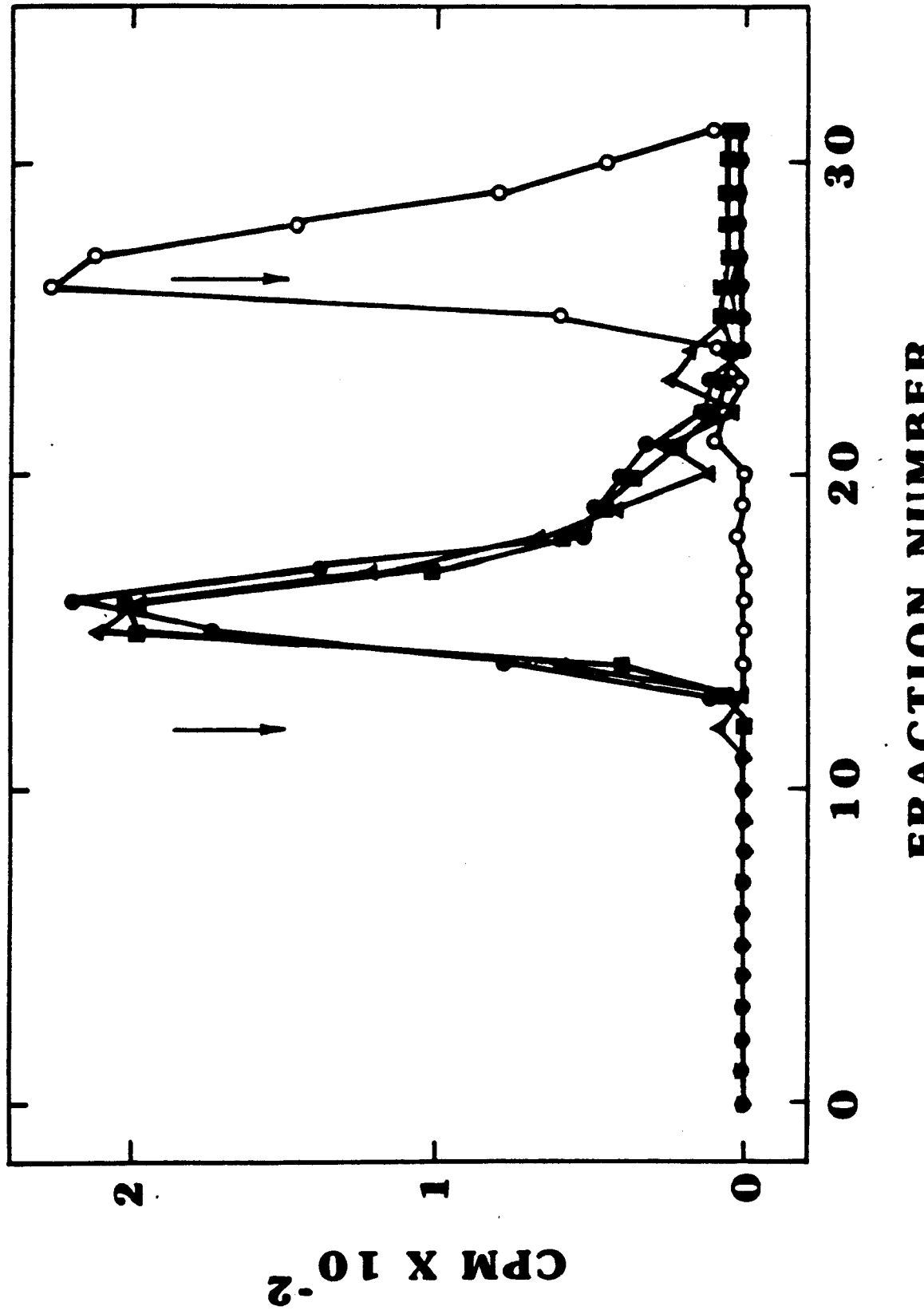

CPM X 10-2
0
1
2
0
10
20
30
FRACTION NUMBER

Figure 16. Molecular sieve chromatography of protein standards prior to and following protease treatments. The arrows indicate the void volume and the included volume, respectively. Enzyme treatments were performed under the same conditions indicated in figure legend 15. (———) untreated material, (- - - -) proteinase k and (●—●—●) pronase E. Peaks 1, 2, 3, 4 and 5 of the solid line correspond to thyroglobulin, IgG, Ovalbumin, Myoglobin and Cyanocobalamin.

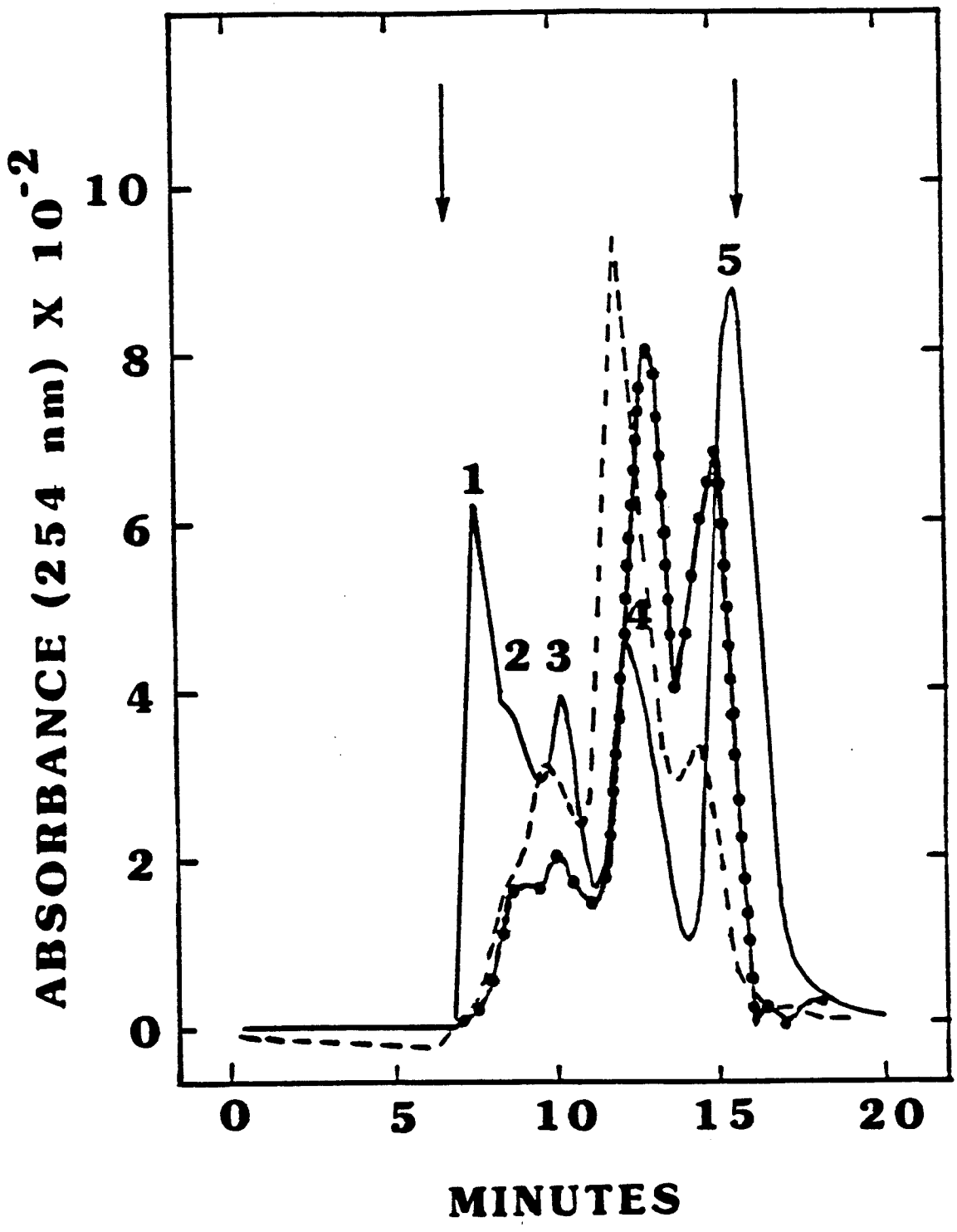

ABSORBANCE (254 nm) X 10^{-2}
10
8
6
4
2
0
0
5
10
15
20
MINUTES
1
2 3
4
5

Figure 17. Polyacrylamide gel electrophoresis of poly(ADP-ribose) following treatment with either proteinase k or trypsin. The electrophoretic conditions were as described in methods except that the gel contained 8 M urea. Lane 1 shows the untreated material. Lane 2 shows treatment with 1 unit of proteinase k at 37°C for 1 hour at pH 7.4. Lane 3 shows treatment with 0.5 units of trypsin at 37°C for 1 hour. Abbreviations: O, origin; XC, xylene cyanol; BPB, bromophenol blue.

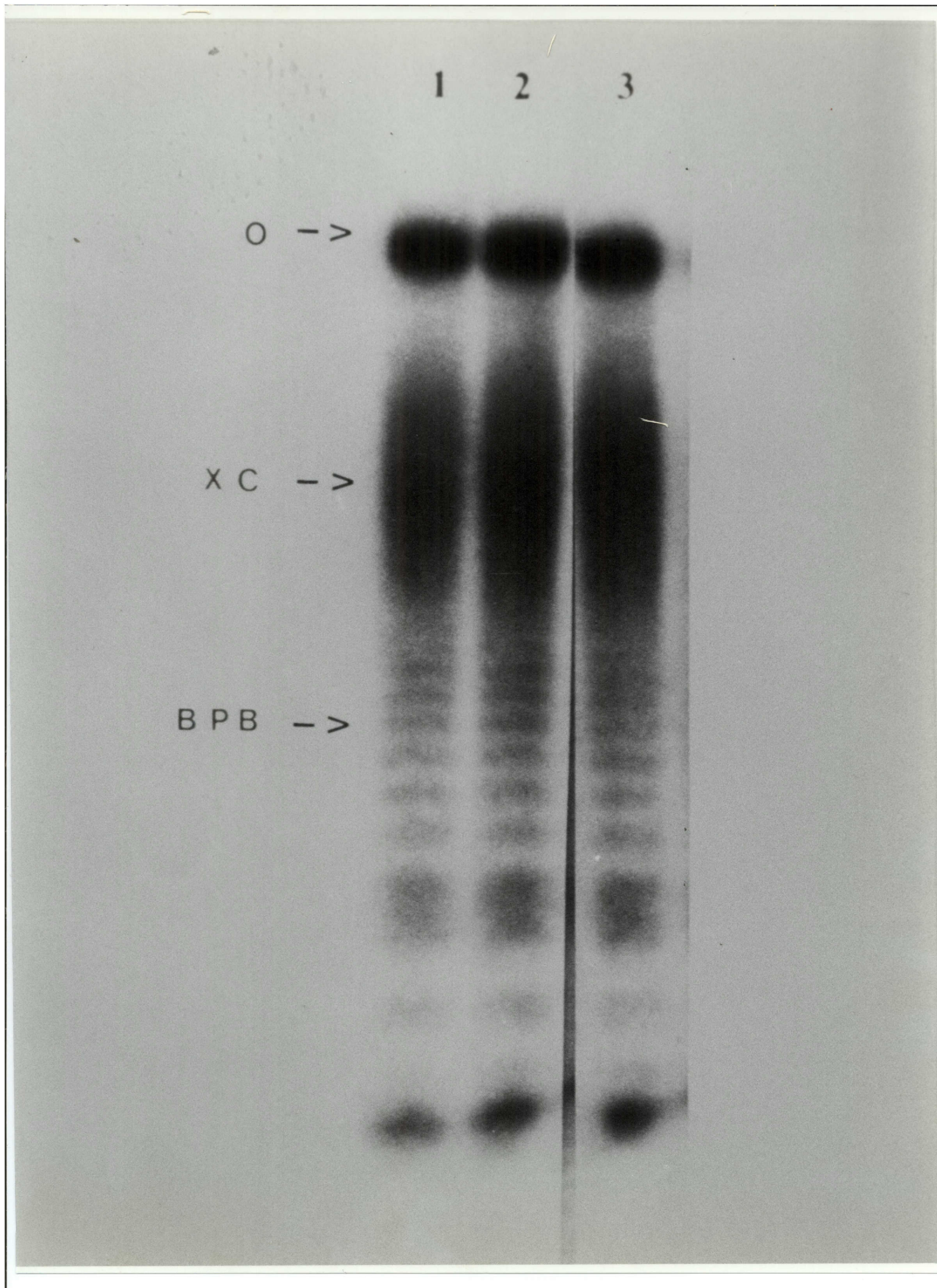
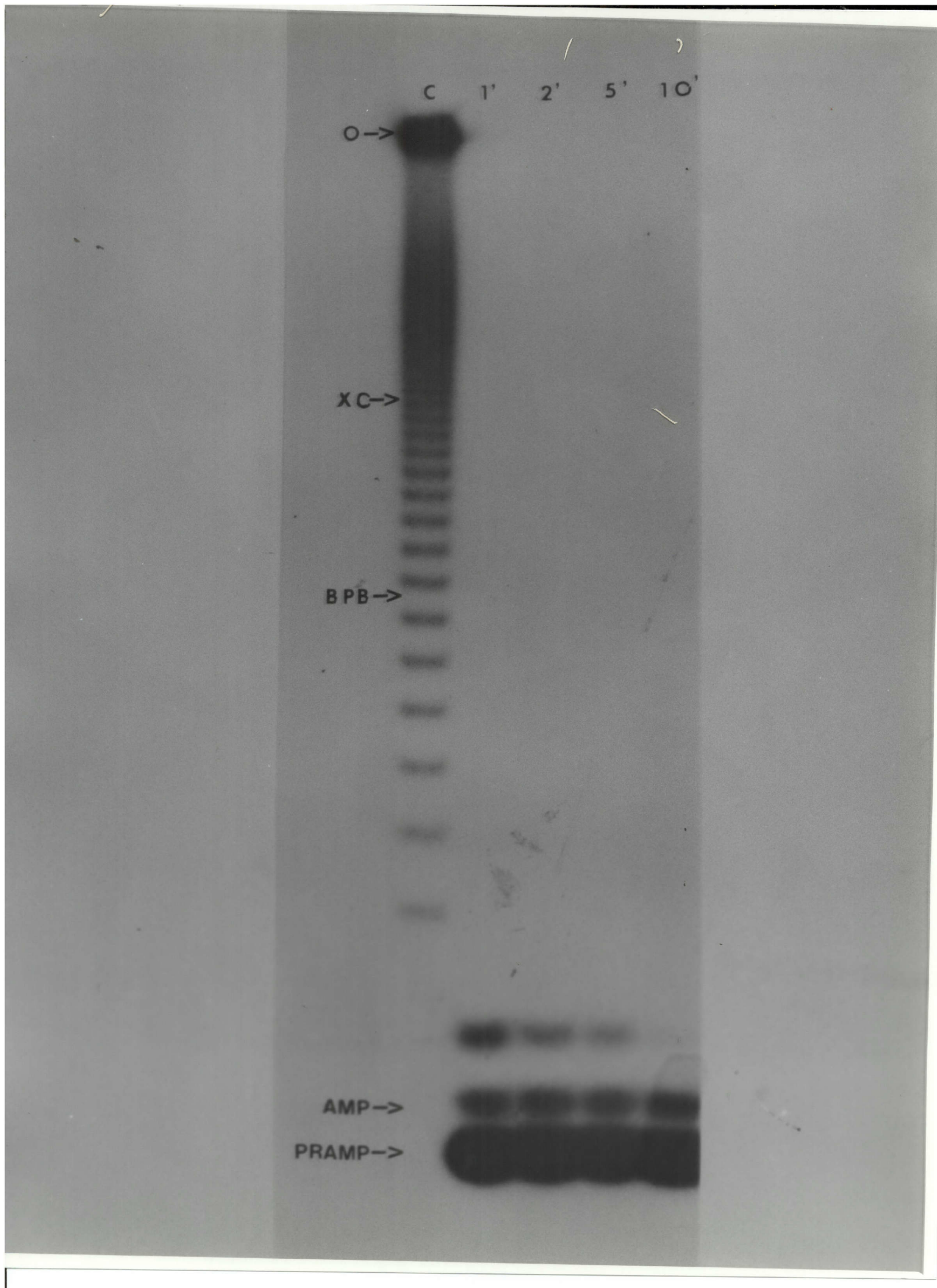
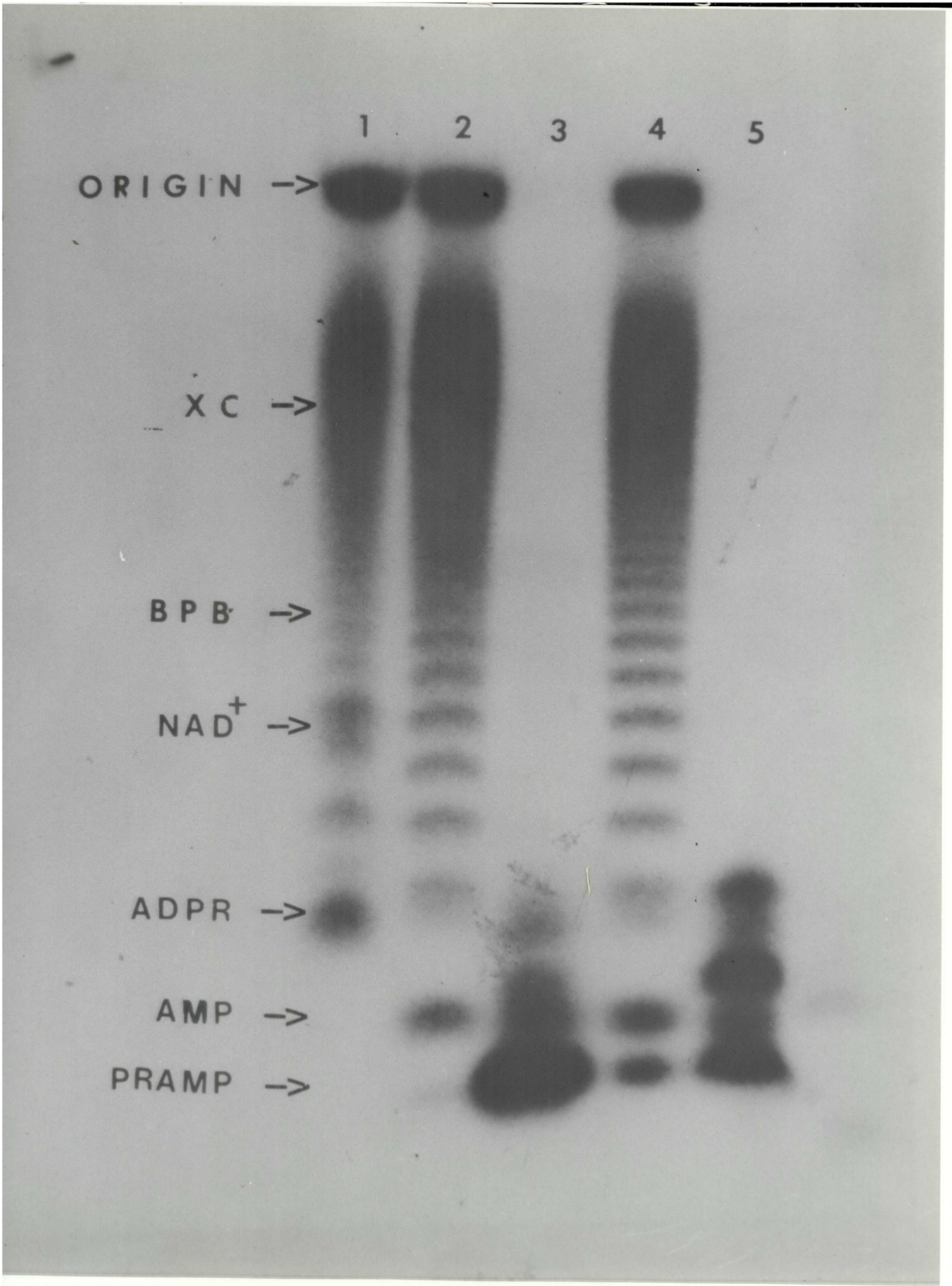

1
2
3
O ->
X C ->
B P B ->

Figure 18. Polyacrylamide gel electrophoresis of poly(ADP-ribose) following SVPD digestion. C, untreated poly(ADP-ribose) (control). 1', 2', 5' and 10' indicate minutes of incubation with 2 units/ml of SVPD at 37°C in the presence of 25 mM NH_4OAc buffer, pH 9.0 and 10 mM $MgCl_2$. Abbreviations: O, origin of application. XC, xylene cyanol. BPB, bromophenol blue.


C
1'
2'
5'
10'
O->
XC->
BPB->
AMP->
PRAMP->

The data presented above demonstrate that material purified by affinity chromatography contained non-protein bound polymers of ADP-ribose. However, since poly(ADP-ribose) is known to be associated with chromatin, it was possible that highly complex polymers were covalently interacting with either RNA or DNA. Thus, to test this possibility, unfractionated polymers of ADP-ribose were subjected to polyacrylamide gel electrophoresis following alkaline treatment. It has been shown by Adamietz and Bredehorst (2) that poly(ADP-ribose) is stable at alkaline pH in the absense of divalent cations, but that the polymer is sensitive to hydrolysis if small amounts of Mg^{++} or other divalent cations are present. Figure 19 shows the stability of the radiolabeled preparations of poly(ADP-ribose) upon treatment with alkali in the presence or absence of Mg^{++} ions. Lane 1 shows the control following incubation at 60°C for 3 hours at neutral pH. Lanes 2 and 3 represent poly(ADP-ribose) treated with either 0.1 N or 0.2 N NaOH for 3 hours at 60°C, in the presence of 20 mM EDTA. It was observed that the profile was unaffected, except that small amounts of contaminating NAD^{+} and ADP-ribose were degraded to AMP. This particular preparation still contained NAD^{+} and ADP-ribose because it had been purified by boronate chromatography following dissolution of the acid insoluble material in 6.0 M Gu.HCl at pH 5.0. Lanes 3 and 5 show that the preparation was completely hydrolyzed to nucleotides

Figure 19. Polyacrylamide gel electrophoresis of unfractionated poly(ADP-ribose) following chemical incubation with alkali in the presence and absence of Mg^{++}. The conditions of electrophoresis were as described in methods except that the gel contained 8 M urea. Lane 1 shows the untreated material (control). Lanes 2 and 4 show material incubated with either 0.1 N or 0.2 N NaOH in the presence of 20 mM EDTA at 60°C for 3 hours. Lanes 3 and 5 show poly(ADP-ribose) treated with either 0.1 N or 0.2 N NaOH at 60°C for 3 hours in the presence of 10 mM MgCl. The positions where NAD_+, ADP-ribose, PRAMP and AMP migrate are also indicated. Abbreviations: XC, xylene cyanol, BPB, bromophenol blue.


1
2
3
4
5
ORIGIN ->
XC ->
BPB ->
NAD+ ->
ADPR ->
AMP ->
PRAMP ->

following treatment with either 0.1 N or 0.2 N NaOH at 60°C for 3 hours in the presence of 10 mM $MgCl_2$. Because RNA is not stable under alkaline conditions even in the absence of Mg^{++}, these data rule out the possibility that poly(ADP-ribose) was covalently associated with significant amounts of RNA. Moreover, the covalent structure of DNA is stable under alkaline conditions even in the presence of Mg^{++}. Thus, it is unlikely that DNA is covalently linked to poly(ADP-ribose).

In summary results from figures 15 to 19 argue that the preparations are composed exclusively of poly(ADP-ribose) and that these preparations are not covalently associated with significant amounts of protein, RNA or DNA.

Analysis of polymer size and complexity.- The size of poly(ADP-ribose) can be determined by the direct analysis of the electrophoretic migration of [^{32}P] radiolabeled material on polyacrylamide gels. However, as mentioned above, the determination of polymer size in polyacrylamide gels of material synthesized in intact cells is complicated by several factors. These include problems of extraction from polyacrylamide gels as well as sensitivity of detection. Further, about 30% of the preparation did not migrate into the gels (figures 7,8,10,13,17,18 and 19). Therefore an alternative methodology was developed. This methodology involves chemical analysis following enzymatic hydrolysis of poly(ADP-ribose) to nucleotides or nucleosides.

Digestion of poly(ADP-ribose) with SVPD results in the generation of the unique nucleotides PRAMP, and $(PR)_2AMP$ from linear internal and branched residues, respectively as well as AMP from terminal residues. Thus, this allows the selective quantification of [^{3}H] and [^{32}P] radiolabeled nucleotides with high sensitivity. Figure 20 depicts a scheme for the enzymatic degradation of a branched polymer of ADP-ribose to nucleotides. Figure 21 shows the structures of the respective digestion products AMP, PRAMP and $(PR)_2AMP$. The accurate determination of the relative amounts of the nucletides by chemical analysis allows the calculation of the average polymer size and the polymer complexity using the mathematical formulas described below.

The average polymer size of a given fraction calculated as minimum average number of ADP-ribose residues per molecule can be obtained from the following equation:

$$\text{Average polymer size} = \frac{AMP + PRAMP + (PR)_2AMP}{AMP - (PR)_2AMP}$$

The average number of points of branching per molecule can be obtained from the following equation:

Figure 20. Enzymatic hydrolysis of poly(ADP-ribose) with SVPD.

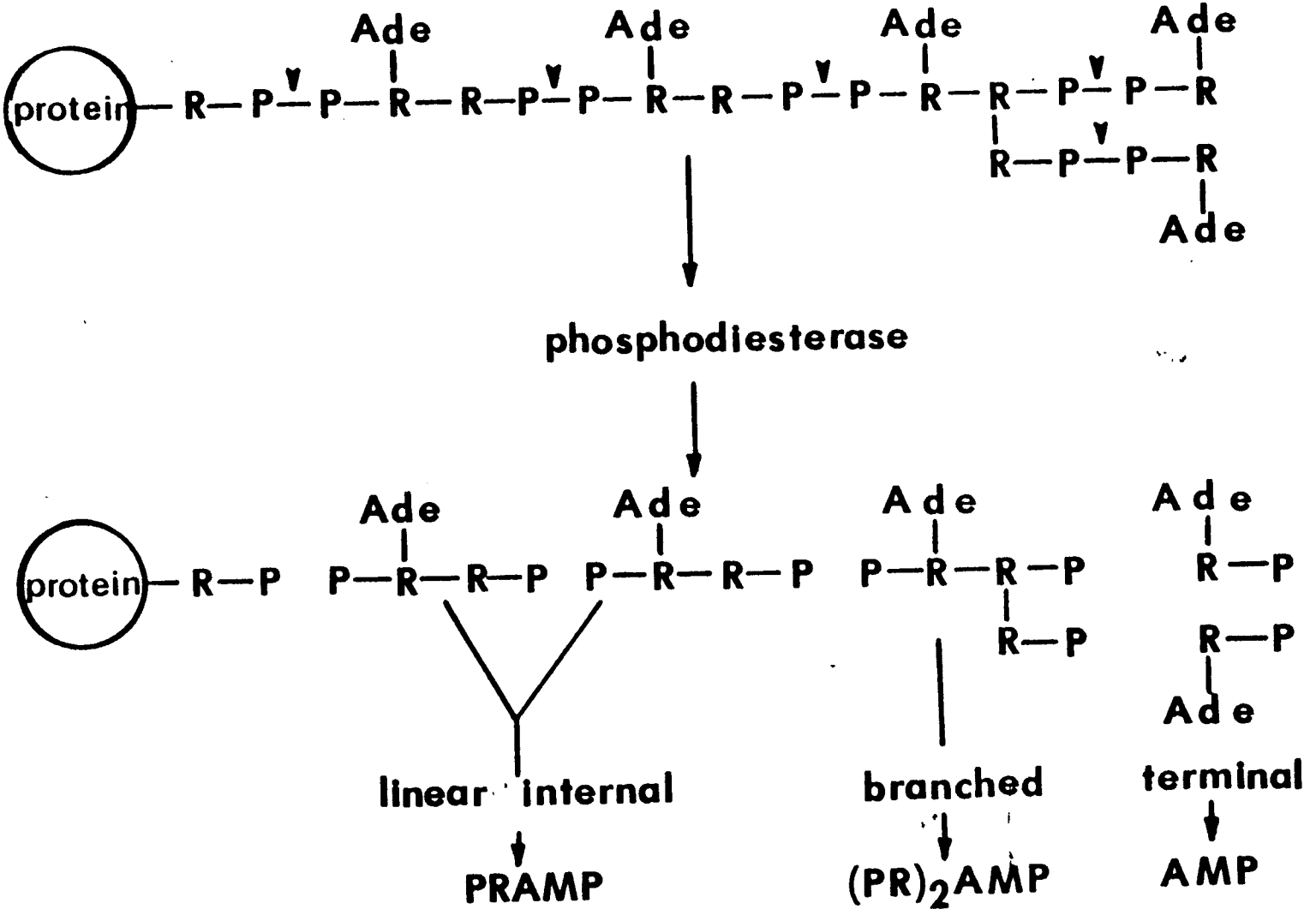
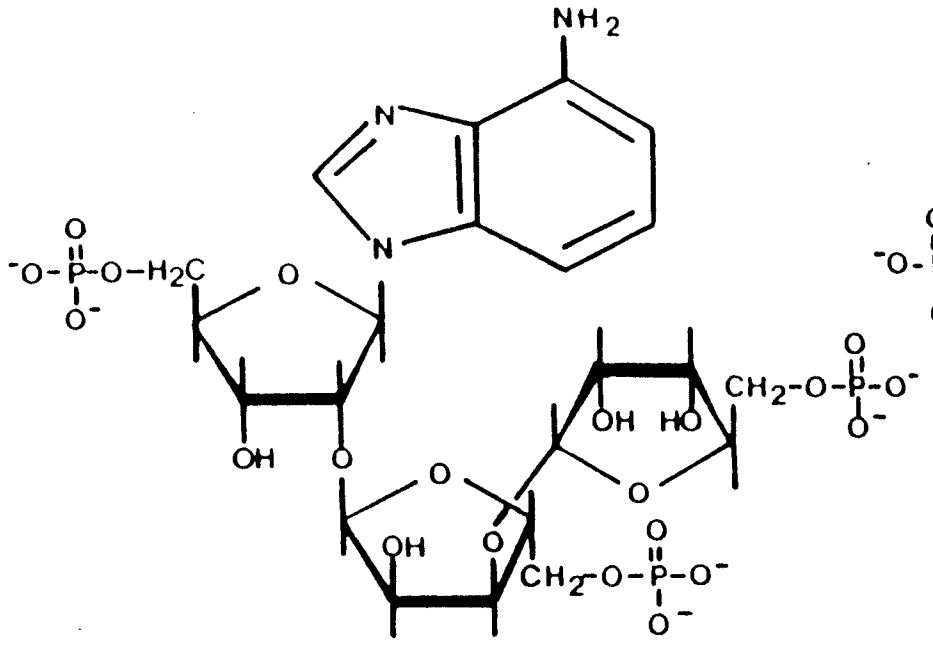
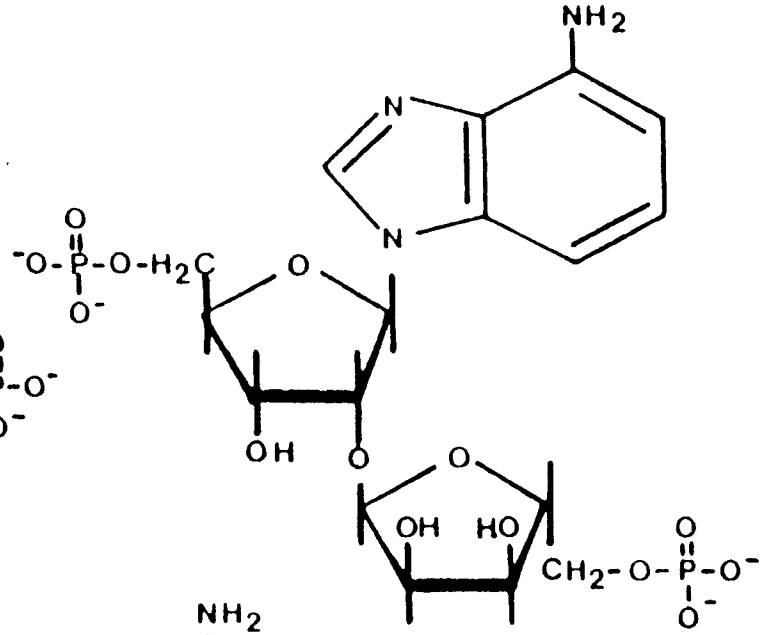

protein
Ade
R—P—P—R—R—P—P—R—R—P—P—R—R—P—P—R
R—P—P—R
phosphodiesterase
protein—R—P
linear internal
PRAMP
branched
(PR)2AMP
terminal
AMP

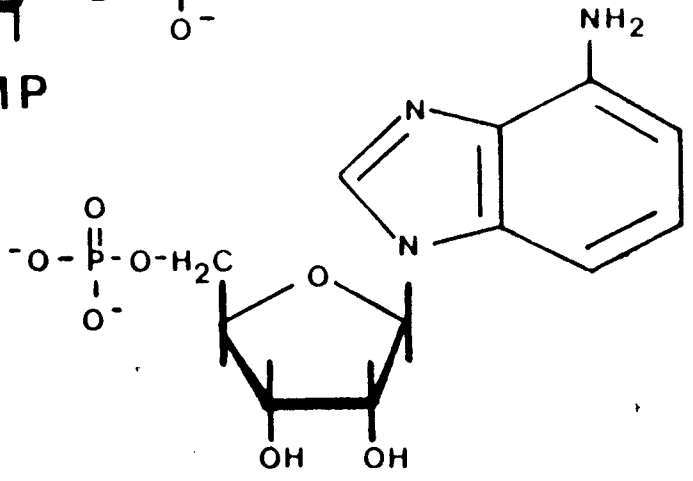
Figure 21. Chemical structures of $(PR)_2AMP$, PRAMP and AMP.



$[PR]_2AMP$



PRAMP



AMP

$$\text{Average number of branching points per molecule} = \frac{(PR)_2AMP}{AMP - (PR)_2AMP}$$

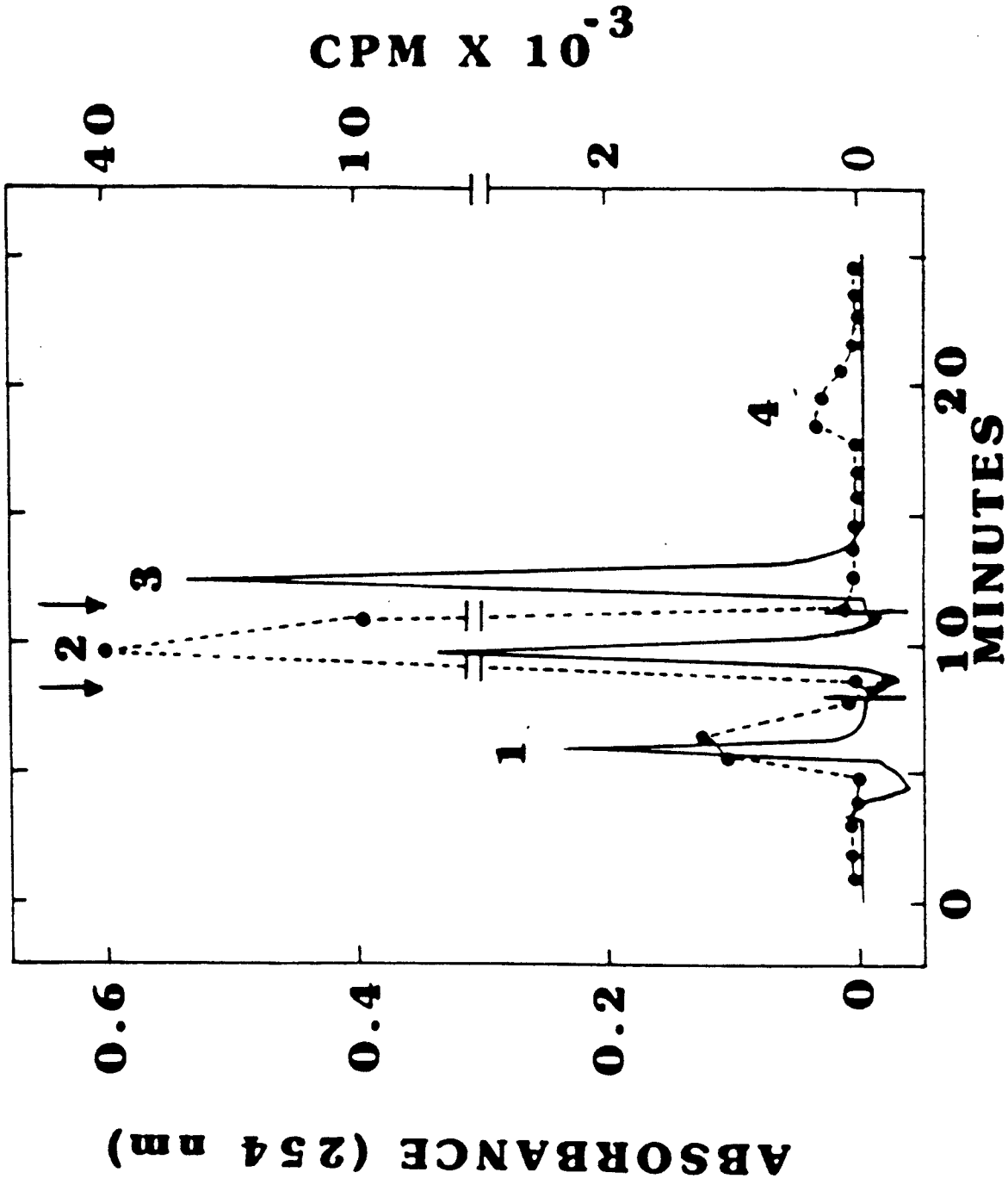
The average polymer size of a linear polymer of ADP-ribose can be calculated by using the following equation:

$$\text{Chain length} = \frac{PRAMP}{AMP} + 1$$

It is important to note that the same basic formulas described above can also be used to determine the size and complexity of poly(ADP-ribose) following digestion to nucleosides. Conversion of poly(ADP-ribose) to nucleosides is achieved following incubation with SVPD and BAP. This generates the unique nucleosides ribosyladenosine (RAdo) and di-ribosyl adenosine (R_2Ado) from linear internal and brnached residues, respectively and adenosine (Ado) from terminal residues. The chemical structures of the nucleosides is the same as the nucleotides (figure 21) except that the 5' terminal phosphates have been removed.

Chemical analysis of either nucleotides or nucleosides was used to determine the average polymer size and complexity of poly(ADP-ribose). A highly sensitive, efficient and fast method was needed to analyze multiple

Figure 22. Analysis of nucleotides by strong anion exchange chromatography following treatment of [^{32}P] poly(ADP-ribose) with SVPD. The arrows indicate a 10-fold increase in absorbance. Peaks 1, 2, 3 and 4 correspond to AMP, PRAMP, ADP and $(PR)_2AMP$ respectively. (———) Absorbance, (●--●) radioactivity.


CPM X 10-3
40
10
2
0
ABSORBANCE (254 nm)
0.6
0.4
0.2
0
MINUTES
0
10
20
1
2
3
4

samples following molecular sieve chromatography. Thus, a chromatographic system that fulfilled these criteria was developed. Figure 22 shows the chemical analysis of nuleotides by strong anion exchange-HPLC. An efficient and fast separation of AMP, PRAMP and $(PR)_2AMP$ was observed.

The unlabeled nucleosides can be determined by fluorescence detection following derivatization to the fluorescent etheno-derivatives with chloroacetaldehyde (85). The etheno nucleosides can be separated by reversed-phase HPLC following purification by boronate chromatography using Affi-gel 601 (77). Figure 23 shows a typical profile of the etheno-nucleosides derived from poly(ADP-ribose). It is important to point out that the total amount of each nucleoside is directly proportional to the fluorescent peak height. To analyze the size and complexity of poly(ADP-ribose) using the formulas described above, it is very important to accurately quantify each nucleoside. Thus, it is necessary that the three etheno-nucleosides are efficiently recovered following affinity chromatography. It was observed that the use of Affi-gel 601 was not satisfactory. Different batches of Affi-gel 601 showed variable recoveries. Therefore, three other commercially available immobilized boronates containing known amounts of boronate were evaluated for the binding efficiency of the etheno-nucleosides. The results are shown in table III. It was observed that PBA-10, the gel with the lowest amount of

Figure 23. HPLC-reversed phase chromatography of etheno-nucleosides derived from poly(ADP-ribose). Nucleosides are obtained by the enzymatic digestion with SVPD and BAP followed by chemical derivatization with chloroacetaldehyde. The arrow indicates a 100-fold increase in sensitivity. Peaks 1, 2 and 3 show the retention times for Ado, RAdo and $(R)_2$Ado respectively.

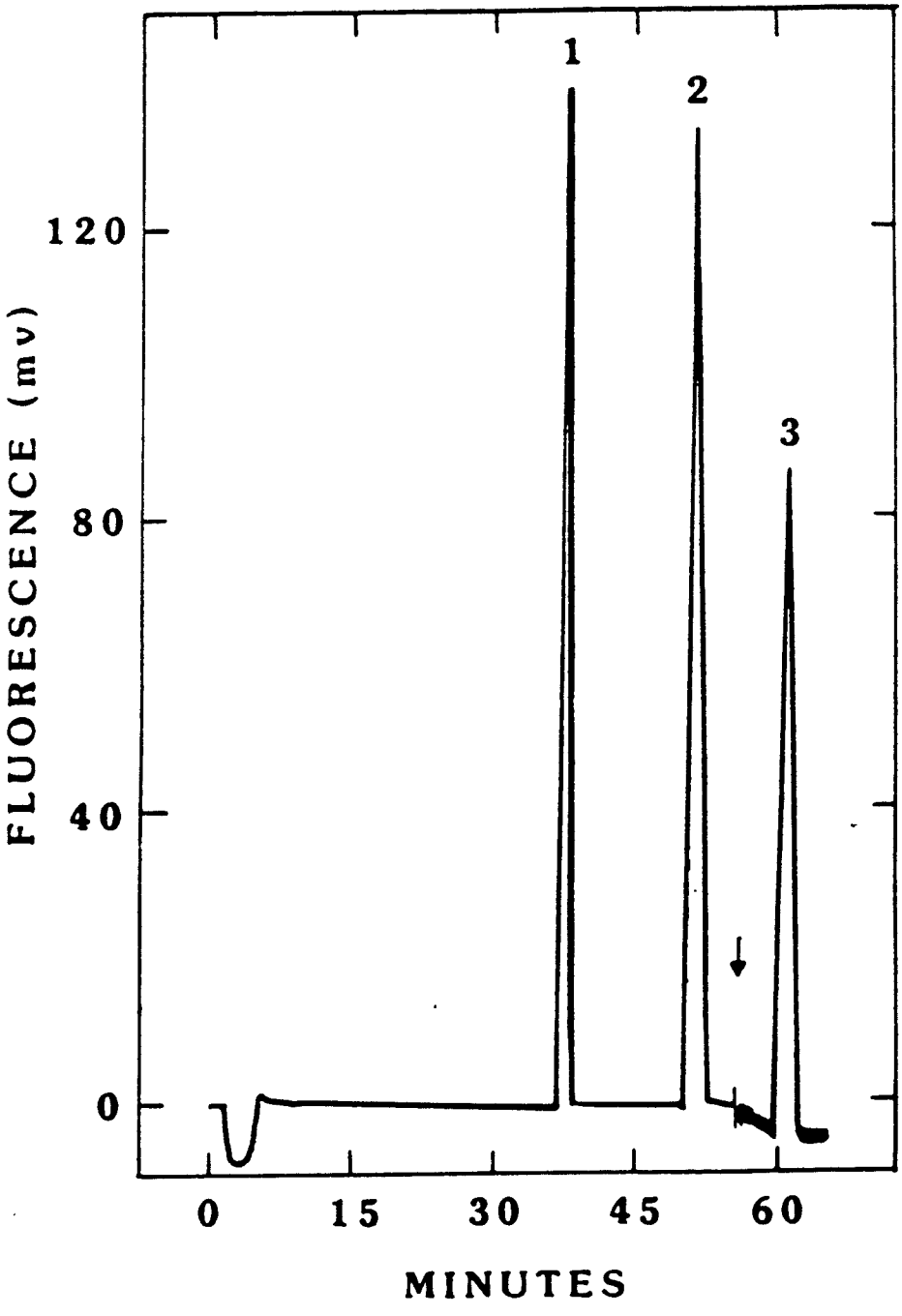

1
2
3
120
80
40
0
FLUORESCENCE (mv)
0
15
30
45
60
MINUTES

TABLE III

Efficiency Of Retention Of Etheno-Nucleosides
By Immobilized Boronates

Boronate	% of total retained		
	Etheno-Ado	Etheno-RAdo	Etheno-R_2Ado
Affi-gel 601	61	37	24
PBA-10	15	<1	<1
PBA-30	90	25	4
PBA-60	84	85	83

Retention efficiency of etheno-nucleosides by the boronate gels was determined by fluorescence detection of the etheno-derivatives prior to and following affinity chromatography.

boronate did not retain the etheno-nucleosides very efficiently. However, PBA-60, the gel which contained the highest amount of boronate, retained all three etheno derivatives efficiently. Thus, PBA-60 was chosen for the purification of etheno-nucleosides prior to HPLC.

Determination of Size and Complexity of Poly(ADP-ribose) in vitro

In order to determine the size and complexity of poly(ADP-ribose) synthesized in nucleotide permeable cells, the fractions collected following molecular sieve chromatography were subjected to digestion with SVPD, followed by analysis of the nucleotides by strong anion exchange-HPLC (figure 22). Table IV shows the relative amounts of each nucleotide in fractions 13 to 23. The average polymer size in each fraction was calculated using the formulae described above and the results are shown in table V. A very broad distribution of sizes was observed that included polymers of an average size of 6 to 190 residues. Figure 24 shows that when the logarithm of the molecular weight was plotted versus the fraction number, a linear relationship was observed. The absolute values of the average polymer size obtained can be validated by comparing the data in table V to the electrophoretic migration of each fraction (figure 13). It was observed that fraction 19 contained a

TABLE IV

Nucleotide Composition Of Fractions Of Poly(ADP-ribose) Synthesized In Permeabilized Cells

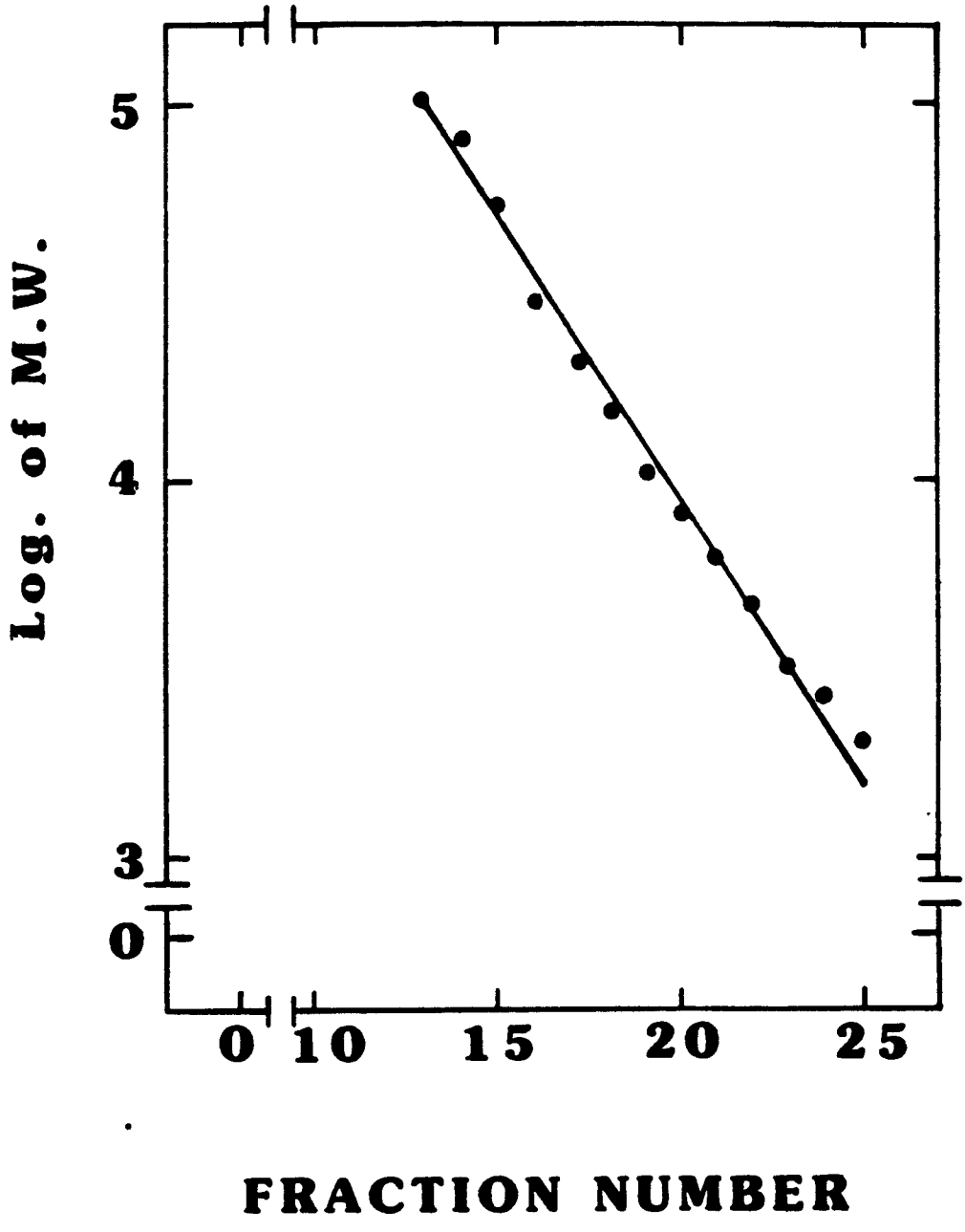
Fraction	cpm			$(PR)_2AMP$
	AMP	PRAMP	$(PR)_2AMP$	% of Total
13	546	14,718	463	2.94
14	2,450	67,452	1,982	2.75
15	7,555	202,905	5,455	2.52
16	8,555	218,305	4,330	1.87
17	11,481	257,165	4,455	1.63
18	14,007	277,525	3,465	1.17
19	17,417	273,182	2,757	0.93
20	19,097	231,250	2,085	0.82
21	20,962	205,687	1,787	0.78
22	20,357	137,637	1,162	0.73
23	18,545	87,037	667	0.62

TABLE V

Analysis Of The Complexity Of Poly(ADP-ribose) Synthesized In Nucleotide Permeable Cells

Fraction	Average Polymer Size	"Average Chain Length"	Points of Branching per Molecule
13	190	28	5.62
14	154	28	4.24
15	102	28	2.59
16	54	26	1.02
17	38	23	0.63
18	28	21	0.32
19	20	17	0.18
20	15	13	0.12
21	12	11	0.09
22	8	8	0.06
23	6	6	0.04

Figure 24. Relationship between the molecular weight and elution volume of poly(ADP-ribose) fractionated by molecular sieve chromatography.


Log. of M.W.
5
4
3
0
0
10
15
20
25
FRACTION NUMBER

population of molecules which migrated on 20% polyacrylamide gels near the dye XC (fig. 13). The calculated average polymer size for this fraction was 20 residues, which agreed perfectly with the analysis on polyacrylamide gels (figure 13). Also shown in table V is a calculation of the average chain length using a method that does not correct for the fact that poly(ADP-ribose) is a branched molecule (121). The estimated polymer size was smaller by as much as 6 to 7 fold (table V) with the maximum average chain length calculated to be approximately 28 residues. The data obtained also permitted the calculation of the average number of branching points per molecule. This calculation is also shown in table V and it was observed that the average number of points of branching increased with increasing polymer size up to more than 6 per molecule. Fractions 13 to 15, material that did not migrate in gels, contained polymers that had multiple points of branching.

Determination of Size and Complexity of Poly(ADP-ribose) in Intact Cells

The methods developed above have been applied to the study of polymer size and complexity in intact cells. The feasability of these studies has been evaluated using radiolabeling techniques as well as fluorescence methods. Conditions that have previously been shown to result in the

alteration of poly(ADP-ribose) metabolism *in vivo* were chosen to determine polymer size and complexity. These included a variety of treatments such as DNA damage using alkylating agents and environmental stresses involving hyperthermia and ethanol treatments.

Analysis of the size and complexity of poly(ADP-ribose) in C3H10T1/2 cells following DNA damage using radiolabeling in vivo.- In order to examine if highly complex polymers were synthesized in intact cells following DNA damage, C3H10T1/2 cells in culture were labeled with [^{3}H] Ade and subsequently subjected to MNNG treatment as indicated in methods. Acid insoluble material was obtained. Approximately 10% of the total radiolabel was recovered with this fraction. [^{3}H] labeled poly(ADP-ribose) was purified from the acid insoluble pellet by affinity chromatography using a boronate resin following treatment with mild alkali in the presence of EDTA at 37°C instead of 60°C to prevent the loss of [^{3}H] label by exchange reactions. Approximately 0.2% of the total radioactivity observed in the acid insoluble pools was recovered in this fraction.

Control experiments were done to determine if any significant amount of radiolabeled RNA contaminated the preparation. Such information was important since RNA would yield AMP upon SVPD treatment. This contamination would lead to an underestimation of the average polymer size. Preparations of [^{3}H] labeled polymer were treated with

alkali in the absence or presence of $MgCl_2$ as described in figure 19 except that incubation was carried out at 37°C overnight. Following this incubation, preparations were neutralized, subjected to treatment with BAP followed by analysis using reversed-phase HPLC, under conditions described in methods. A typical separation of nucleosides by this technique is shown in figure 25. Radiolabeled Ado was only detected in alkaline incubations done in the presence of $MgCl_2$, which would be expected for poly(ADP-ribose), while any radiolabel present in RNA would have also been present in the samples incubated with alkali and EDTA. Thus, it was possible to rule out a significant contamination with RNA.

Figure 26 shows the profile of poly(ADP-ribose) analyzed by molecular sieve chromatography. The better separation of oligonucleotide standards observed in figure 26 as compared to figure 14 is due to using two Bio-Sil TSK-125 columns in series. It was noted that radiolabeled material eluted very close to the void volume. Fractions containing radiolabel were subjected to SVPD digestion and then to nucleotide analysis by strong anion exchange HPLC. Table VI shows the relative amounts of radiolabel of each nucleotide in fractions 24 to 38. Table VII shows the calculated average polymer size and the average number of branching points per molecule. The average polymer size varied from 11 to 67 residues. The largest polymers

Figure 25. HPLC- reversed phase separation of nucleosides following enzymatic digestion of poly(ADP-ribose) with SVPD and BAP. The arrows indicate from left to right a 10 and 100 fold increase in sensitivity, respectively. Peaks 1, 2 and 3 correspond to Ado, RAdo and $(R)_2$Ado, respectively.

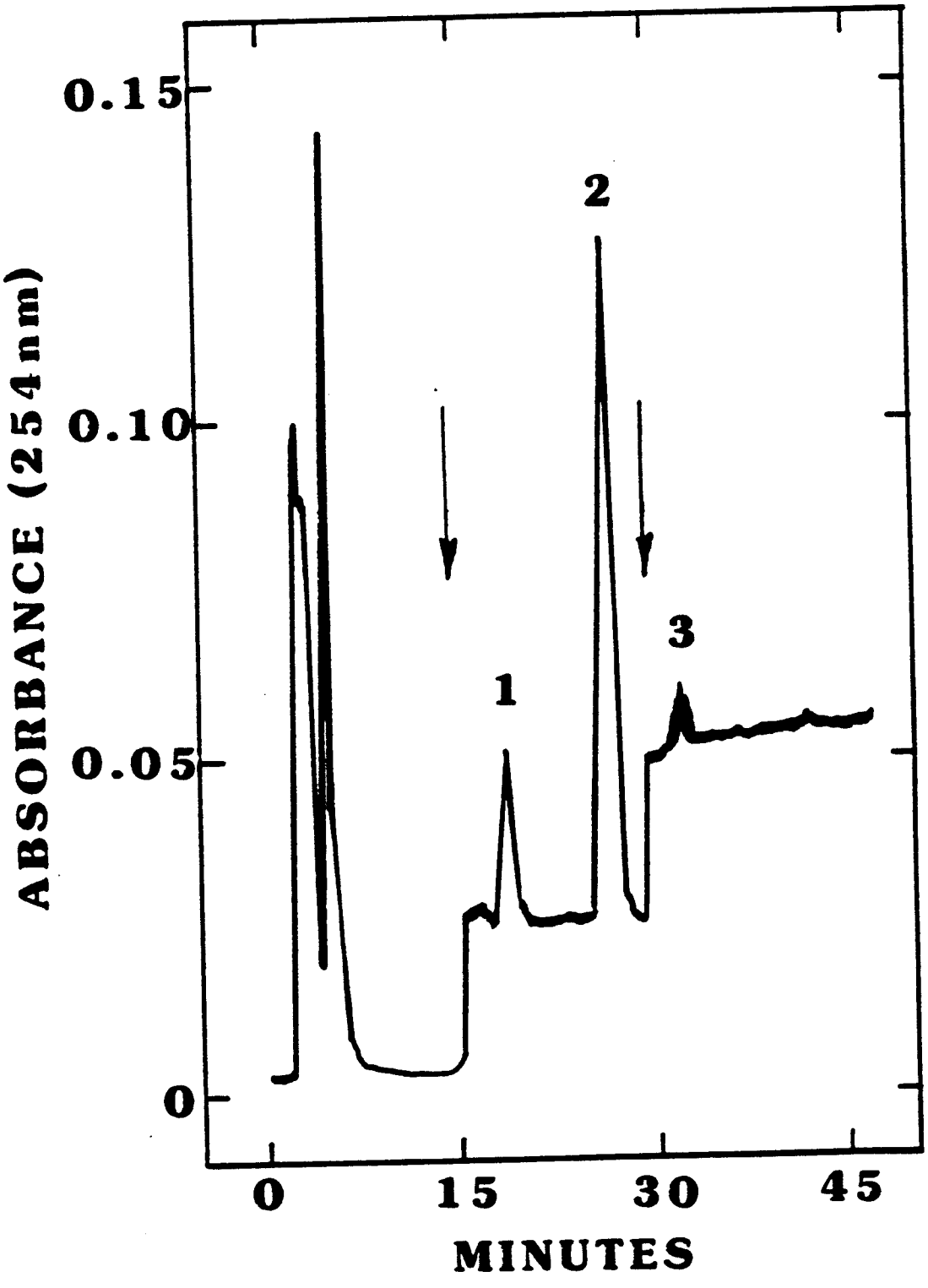

0.15
0.10
0.05
0
ABSORBANCE (254nm)
1
2
3
0
15
30
45
MINUTES

Figure 26. Molecular sieve chromatography of [^{3}H] poly(ADP-ribose)synthesized in C3H10T1/2 cells following DNA damage. Two TSK-125 columns in series were used. The arrows indicate the void volume and included volume respectively. (●——●) Radioactivity, (----) absorbance. Peaks 1, 2, 3 and 4 correspond to poly (A), $(Ap)_9A$, $(Ap)_5A$ and AMP, respectively.

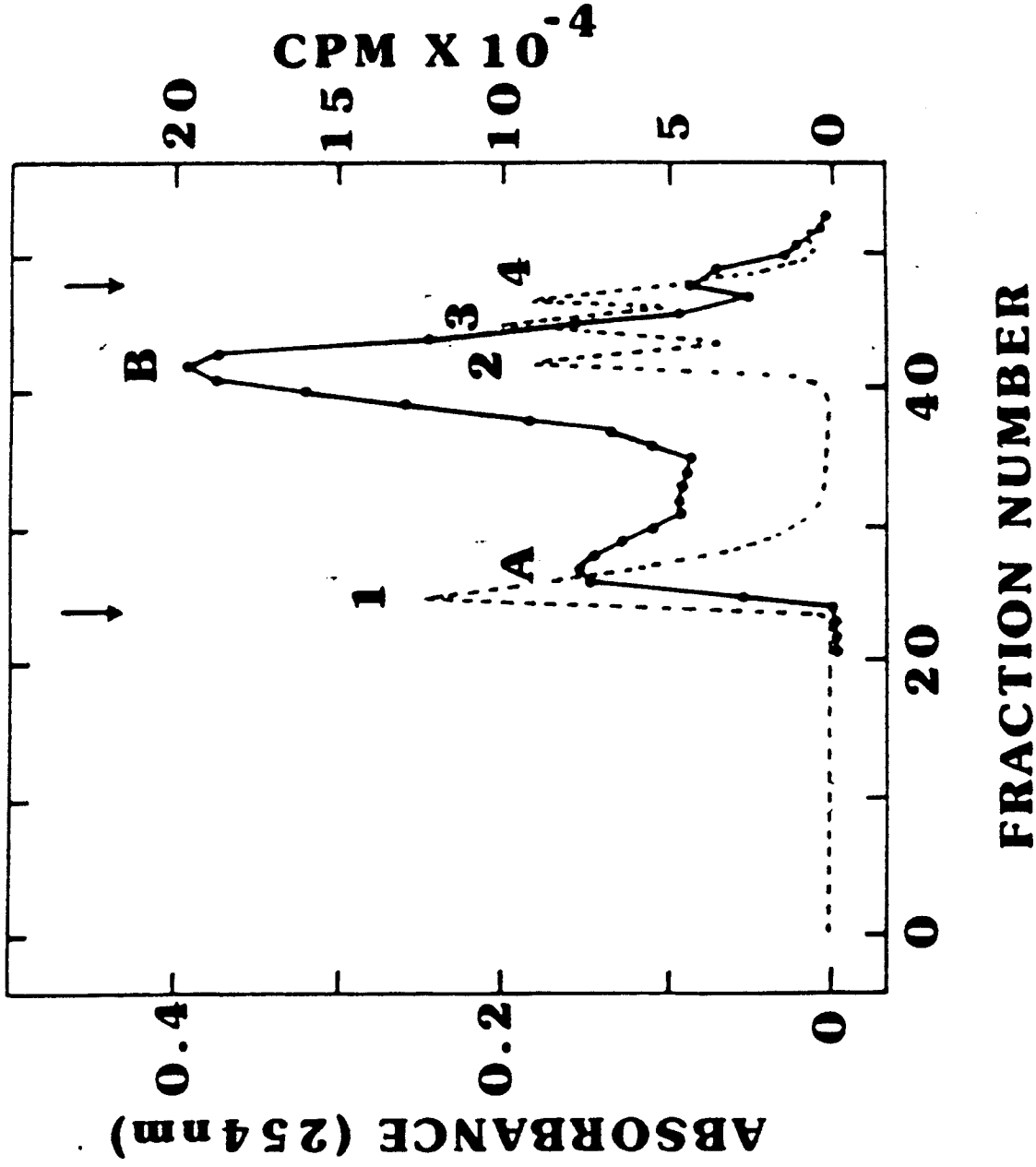

CPM X 10^-4
20
15
10
5
0
B
A
1
2
3
4
0.4
0.2
0
ABSORBANCE (254nm)
0
20
40
FRACTION NUMBER

Table VI

Nucleotide Composition Of Poly(ADP-ribose) Fractions From Polymer Synthesized In Intact Cells Following DNA Damage

Fraction	cpm			$(PR)_2AMP$ % of Total
	AMP	PRAMP	$(PR)_2AMP$	
24	345	6,568	239	3.34
25	761	16,168	500	2.86
26	833	16,840	515	2.83
27	539	11,101	327	2.73
28	776	14,355	420	2.70
29	510	9,514	275	2.67
30	392	7,113	137	1.79
31	432	7,973	141	1.64
32	397	7,418	130	1.63
33	401	6,288	110	1.61
34	414	6,175	103	1.53
35	584	6,688	105	1.42
36	830	9,562	90	0.95
37	1,718	12,506	118	0.82
38	1,293	11,630	105	0.80

Table VII

Analysis of the Complexity of Poly(ADP-ribose) Synthesized in Intact Cells Following DNA Damage

Fraction	Average Polymer Size	Points Of Branching per Molecule
24	67	2.20
25	61	1.90
26	57	1.60
27	56	1.50
28	44	1.20
29	44	1.20
30	30	0.53
31	29	0.48
32	29	0.48
33	23	0.38
34	21	0.33
35	15	0.22
36	14	0.12
37	14	0.07
38	11	0.08

observed contained at least 2 branching points per molecule. It is interesting to note that fractions contained in peak A had more than one point of branching per molecule.

Analysis of the size and complexity of poly(ADP-ribose) in C3H10T1/2 cells following hyperthermia and DNA damage utilizing in vivo radiolabeling.- Recent studies have demonstrated that hyperthermic treatment results in an alteration of poly(ADP-ribose) metabolism (83,89). Thus, polymers synthesized in response to MNNG following hyperthermia were also characterized. Cells were subjected to incubation with 64 μM MNNG for 20 min at 37°C following acute heat shock at 45°C for 30 min and the acid insoluble pools were obtained. Polymers of ADP-ribose were purified by boronate chromatography and the boronate eluate accounted for approximately 1% of the total radioctivity observed in the acid insoluble pools.

Poly(ADP-ribose) preparations were fractionated according to size by molecular sieve chromatography. Figure 27 shows the profile of [^{3}H] radiolabel. Again two apparent classes of polymers were observed (peaks A and B). Each fraction containing radiolabel was subjected to strong anion exchange-HPLC following SVPD treatment. The relative amounts of each nucleotide in fractions 24 to 38 are shown in table VIII. Table IX shows the calculated average polymer size as well as the number of branching points per molecule. The average polymer size increased from 9 to 244

Figure 27. Molecular sieve chromatography of [^{3}H] poly(ADP-ribose) synthesized in C3H10T1/2 cells following hyperthermia and DNA damage. Two TSK-125 columns in series were used. The arrows denote the void volume and included volume respectively. (●—●—●) Radioactivity, (— — —) absorbance. Peaks 1, 2, 3 and 4 represent poly (A), $(Ap)_9A$, $(Ap)_5A$, and AMP.

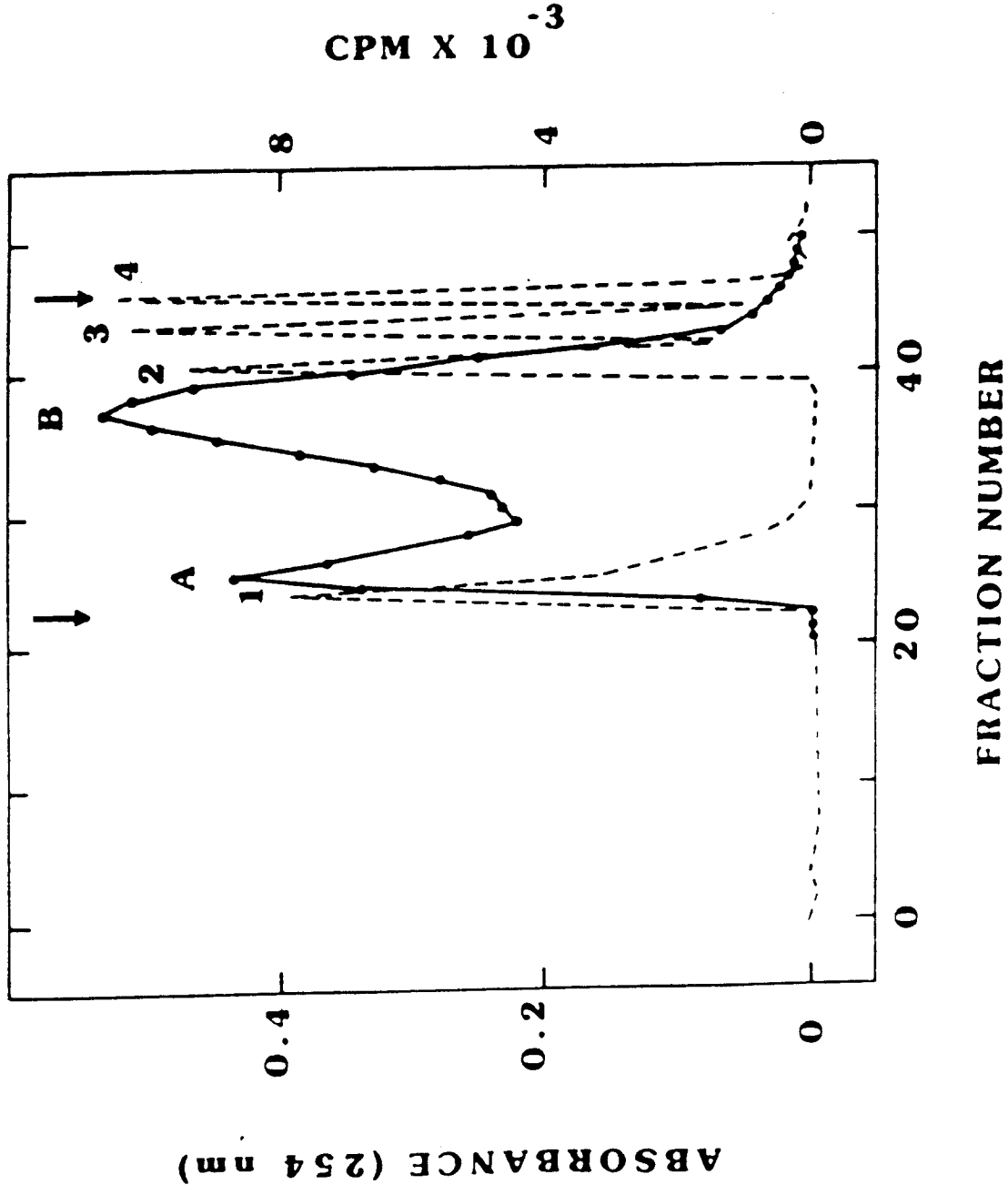

CPM X 10 -3
8
4
0
4
3
2
B
A
1
0
20
40
FRACTION NUMBER
0.4
0.2
0
ABSORBANCE (254 nm)

Table VIII

Nucleotide Composition Of Poly(ADP-ribose) Fractions Of Polymer Synthesized In Intact Cells Following Hyperthermia And DNA Damage

Fraction	cpm			$(PR)_2AMP$ % of Total
	AMP	PRAMP	$(PR)_2AMP$	
24	524	13,931	463	3.10
25	3,064	76,301	2,497	3.04
26	3,449	81,687	2,689	3.06
27	3,211	74,685	2,073	2.59
28	2,691	56,296	1,520	2.51
29	2,280	45,534	1,140	2.32
30	2,420	45,440	998	2.04
31	2,427	42,165	844	1.85
32	2,649	44,120	718	1.51
33	3,583	49,925	763	1.40
34	4,683	57,048	759	1.21
35	6,045	67,842	755	1.01
36	7,198	76,474	743	0.88
37	10,230	84,932	796	0.82
38	13,378	90,118	839	0.80

Table IX

Analysis Of The Complexity Of Poly(ADP-ribose) Synthesized In Intact Cells Following Hyperthermia And DNA Damage

Fraction	Average Polymer Size	Points of Branching per Molecule
24	244	6.6
25	145	4.4
26	115	3.5
27	70	1.8
28	52	1.3
29	43	1.0
30	34	0.7
31	29	0.5
32	25	0.4
33	19	0.2
34	16	0.2
35	14	0.1
36	13	0.1
37	10	0.1
38	9	0.1

residues. The large polymers contained 6 to 7 points of branching per molecule. Again, all of the fractions contained in peak A of figure 27 contained polymers with multiple branching points.

Analysis of the size and complexity of poly(ADP-ribose) utilizing fluorescence methods.- It should be of interest to determine polymer size and complexity under no radiolabeling conditions when analysing tissues. Therefore, fluorescence methodology (85) was evaluated to determine the complexity of poly(ADP-ribose). Initial experiments were done to determine total polymer levels following treatment with a combination of stresses. Figure 28 shows the response of SVT2 cells in culture to different amounts of MNNG at 37°C in the presence of 1% ethanol following hyperthermia at 45°C for 30 min in the presence of 1% ethanol. The levels of poly(ADP-ribose) were stimulated up to 1000-fold over basal levels at 64 µM MNNG. Concentrations above 64 µM MNNG did not result in further increases in polymer levels. Figure 29 shows the time dependence of polymer accumulation following 64 µM of MNNG at 37°C in 1% ethanol after treatment at 45°C for 30 min in the presence of 1% ethanol. The polymer levels were elevated to more than 2000-fold the basal levels at 20 min following addition of MNNG. These conditions were used for the analysis of poly(ADP-ribose). Purified polymers of ADP-ribose were subjected to molecular sieve chromatography using a TSK-125

Figure 28. Effect of MNNG concentration on the accumulation of poly(ADP-ribose) in SVT2 cells following hyperthermia. Cells in culture were treated for 20 min with MNNG in the presence of 1% ethanol following incubation at 45°C for 30 min, in 1% ethanol.

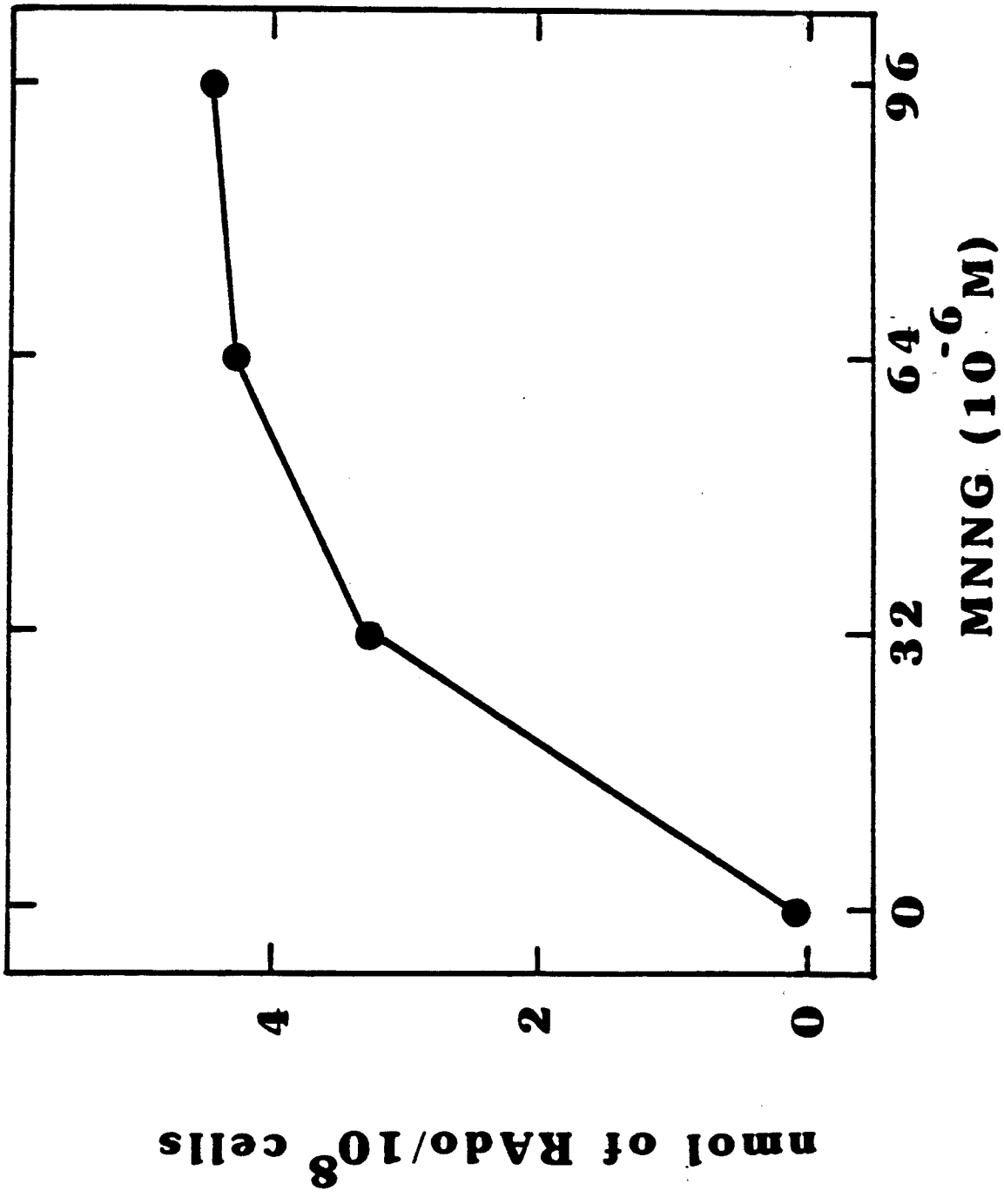

nmol of RAdo/10^8 cells
0
2
4
MNNG (10^-6 M)
0
32
64
96

Figure 29. Time dependance of the accumulation of poly(ADP-ribose) in SVT2 cells in the presence of MNNG following hyperthermia. Cells in tissue were treated with 64 μM MNNG and 1% ethanol following hyperthermic treatment at 45°C for 30 min in 1% ethanol.

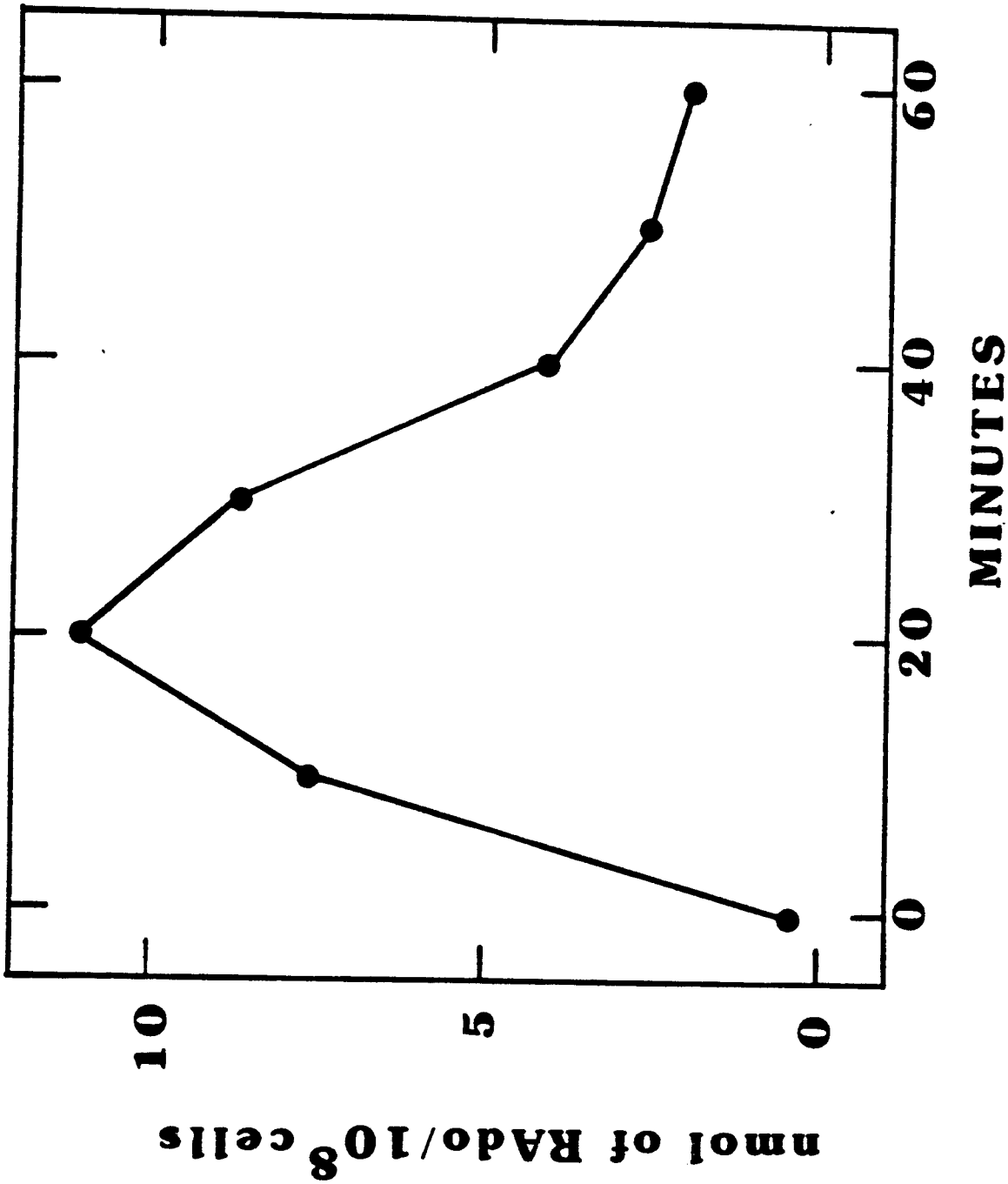
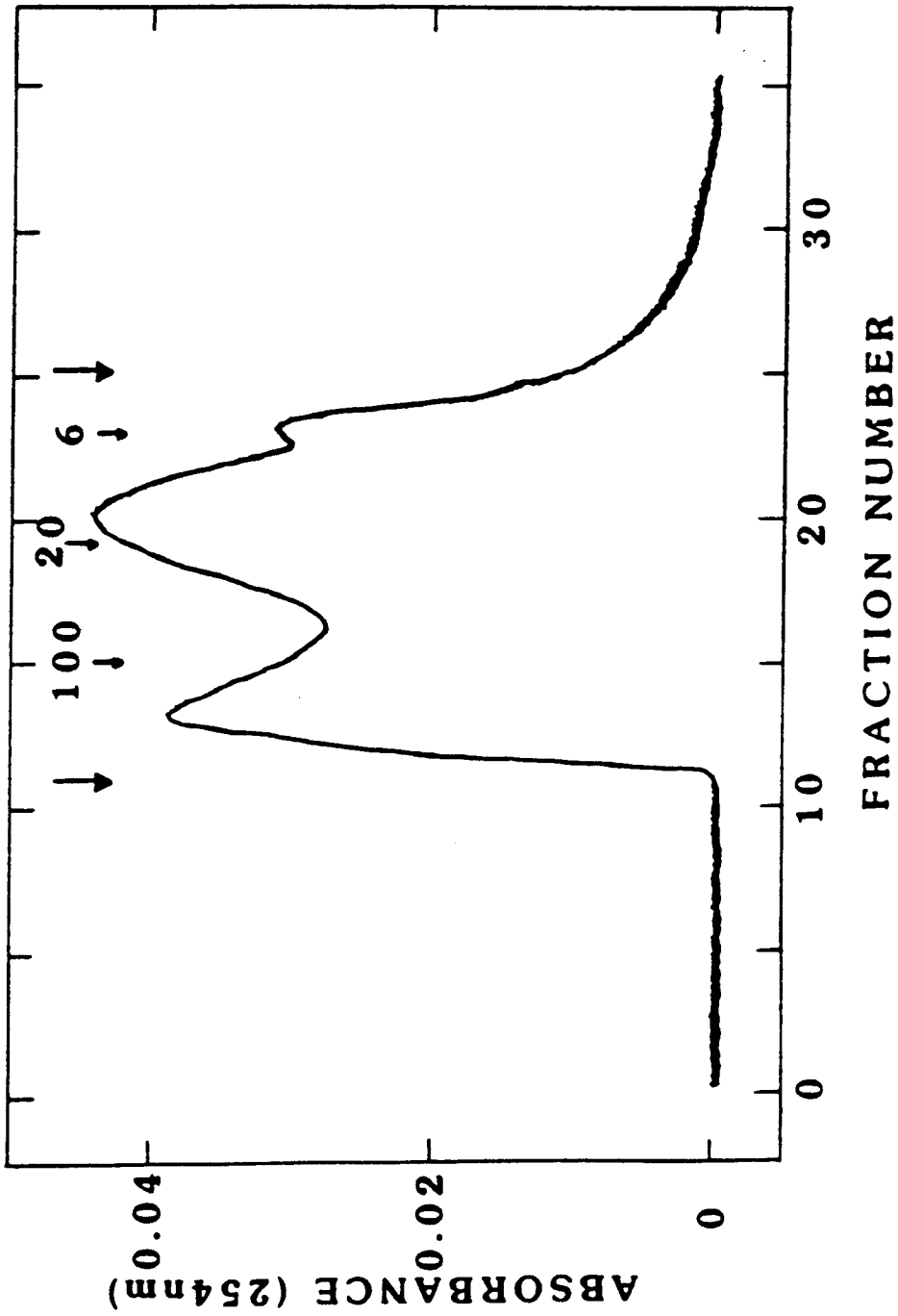

nmol of RAdo/10^8 cells
0
5
10
0
20
40
60
MINUTES

Figure 30. Molecular sieve chromatography of poly(ADP-ribose) synthesized in SVT2 cells following stress. Cells were treated with hyperthermia at 45°C and 1% ethanol followed by DNA damage with 64 μM MNNG in the presence of 1% ethanol. The arrows indicate void volume, elution of markers of known size and included volume, respectively.


ABSORBANCE (254nm)
0
0.02
0.04
0
10
20
30
FRACTION NUMBER
100
20
6

column and absorbance at 254 nm was monitored as indicated in methods. Figure 30 shows that three peaks were observed. One peak eluted close to the void volume suggesting that highly complex polymers of poly(ADP-ribose) are generated in intact cells. Fractions 13 to 26 were subjected to treatment with SVPD and BAP. This was followed by chemical conversion of nucleosides to highly fluorescent etheno derivatives which were determined by reversed-phase HPLC (figure 23). Table X shows the quantification of fluorescence of the etheno nucleosides in these fractions. Table XI shows the calculated average polymer size in each fraction as well as the average number of points of branching per molecule. Polymers of 2 up to 174 residues in size were noted.

Studies on the Mechanism of Branching of Poly(ADP-ribose)

It was noted with interest that oligomers of poly(ADP-ribose) of less than 20 residues in size synthesized in nucleotide permeable cells contained significant amounts of branching. Figure 31 shows that poly(ADP-ribose) from nucleotide permeable SVT2 cells is relatively stable. Most of the radiolabeled material remained acid insoluble when cold NAD^+ was used to substitute labeled NAD^+ in the incubation mixture. Thus, the small polymers detected under

TABLE X

Nucleoside Composition Of Poly(ADP-ribose) Synthesized In SVT2 Cells Following Stress

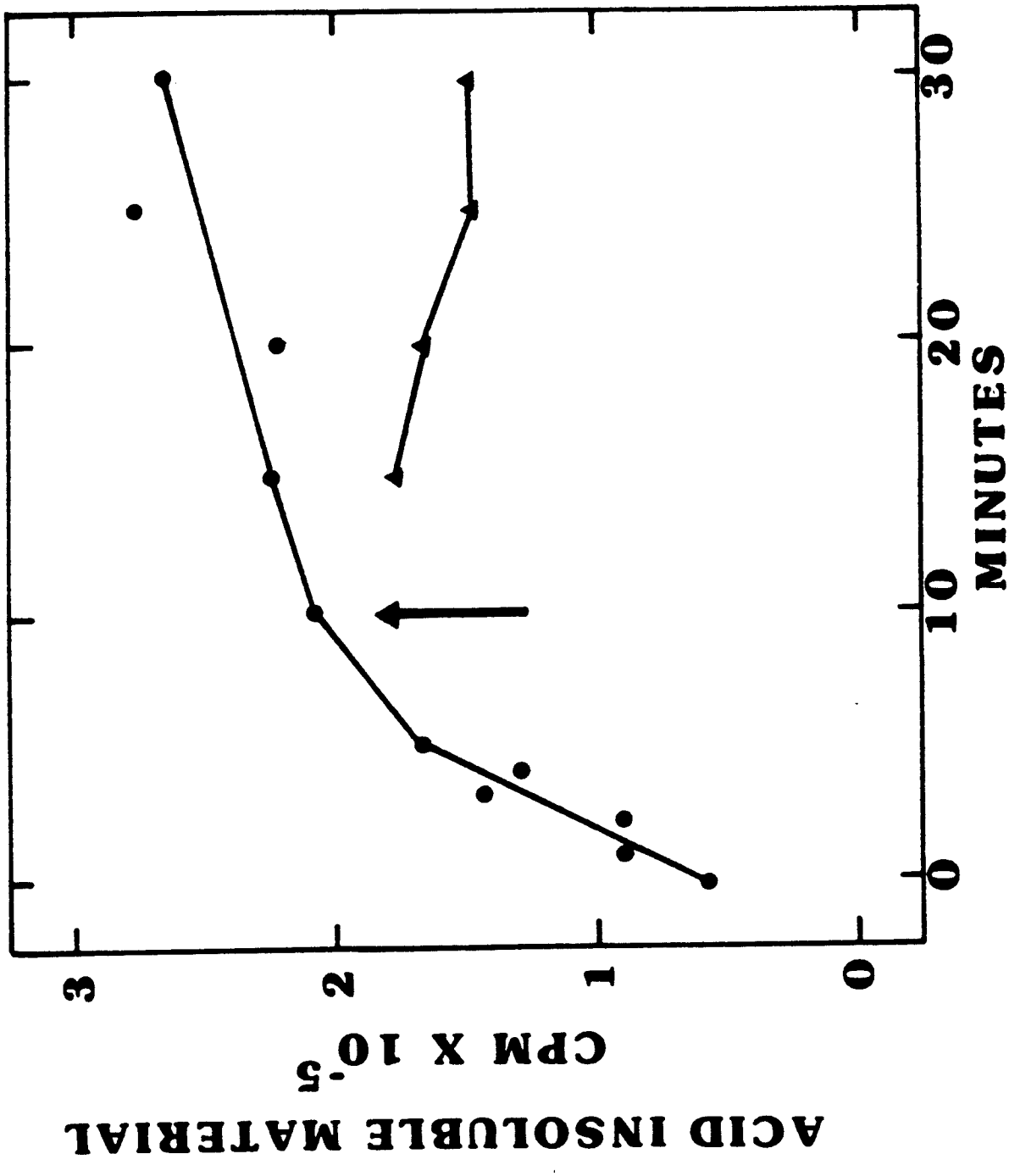
Fraction	Fluorescence (mv)			Etheno-R_2Ado % of Total
	ε-Ado	ε-RAdo	ε-R_2Ado	
13	0.6	25.0	0.50	1.9
14	4.4	105.4	2.45	2.2
15	5.7	122.6	2.70	2.1
16	5.0	126.3	2.55	1.9
17	4.1	113.9	1.95	1.6
18	7.5	143.2	1.85	1.2
19	15.9	206.0	1.90	0.8
20	25.6	239.0	1.70	0.6
21	36.6	266.0	1.60	0.5
22	30.4	178.2	0.85	0.4
23	26.5	121.5	0.55	0.4
24	43.5	90.9	0.40	0.3

TABLE XI

Analysis Of The Complexity Of Poly(ADP-ribose) Synthesized In SVT2 Cells Following Stress

Fraction	Average Polymer Size	Points Of Branching per Molecule
13	174	3.33
14	58	1.25
15	44	1.00
16	55	1.04
17	56	0.99
18	27	0.32
19	16	0.13
20	11	0.06
21	9	0.04
22	7	0.03
23	6	0.01
24	3	--

Figure 31. Stability of poly(ADP-ribose) synthesized in nucleotide permeable cells. The arrow indicates the time at which the labeled NAD^+ in the incubation mixture was substituted with unlabeled NAD^+. (●——●) Incubation in the presence of [^{32}P] NAD^+, (▲——▲) incubation in the presence of unlabeled NAD^+.


ACID INSOLUBLE MATERIAL
CPM X 10^{-5}
0
1
2
3
0
10
20
30
MINUTES

these conditions should correspond to intermediates in synthesis and not degradation products of highly complex polymers of ADP-ribose. In order to determine whether oligomers of poly(ADP-ribose) synthesized in nucleotide permeable cells corresponded to intermediates of synthesis or degradation products of complex polymers of ADP-ribose, a portion of the acid precipitable material from each of the samples collected in the label chase experiment was subjected to polyacrylamide gel electrophoresis. The results are shown in figure 32. The amounts of all polymer sizes increased with the time of incubation in the presence of [^{32}P] labeled NAD^+. However, when the unlabeled NAD^+ was used to chase the labeled material, bands that correspond to small oligomers of ADP-ribose decreased in intensity, while the relative amounts of radioactivity at the origin of the gels, which corresponds to the highly complex polymers of ADP-ribose, increased in intensity. The polyacrylamide gel was sliced in different fragments for the quantification of the radioactivity. One of these fragments contained oligomers of ADP-ribose of 2 to 17 residues in size, the other fragment corresponded to the material at the origin of the gel. Figure 33 shows that synthesis of total polymers of ADP-ribose increased with time. In contrast, the radioactivity associated with oligomers of ADP-ribose (2 to 17 residues) reached a constant level after 10 min of incubation. The amount of of highly complex polymers

Figure 32. Stability of radiolabeled poly(ADP-ribose) synthesized in nucleotide permeable cells as judged by polyacrylamide gel electrophoresis. The numbers at the top of each lane indicate time of incubation. The signs in parenthesis indicate presence or absense of radiolabeled NAD^+. Abbreviations: O, origin. XC, xylene cyanol. BPB, bromophenol blue.

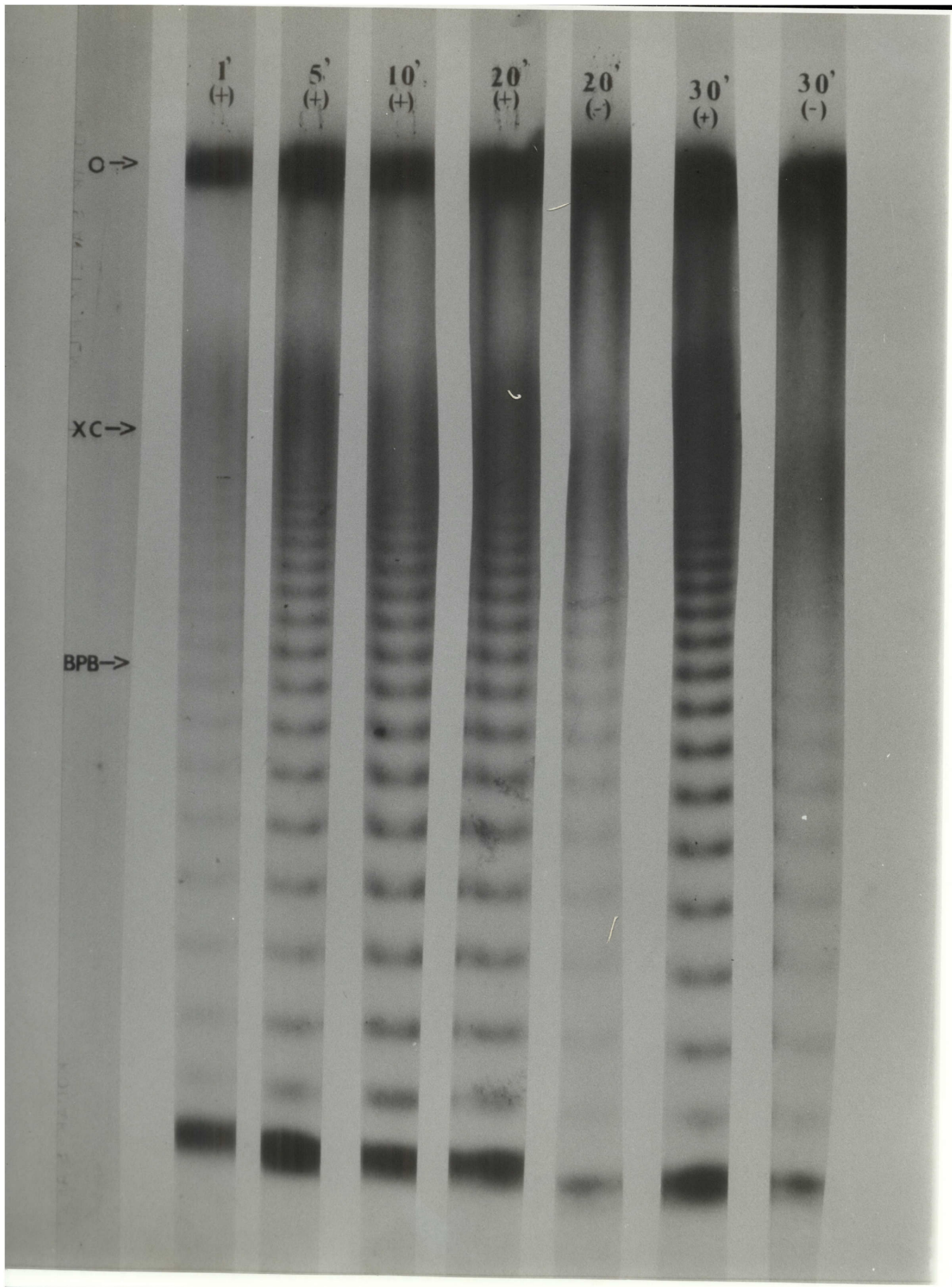
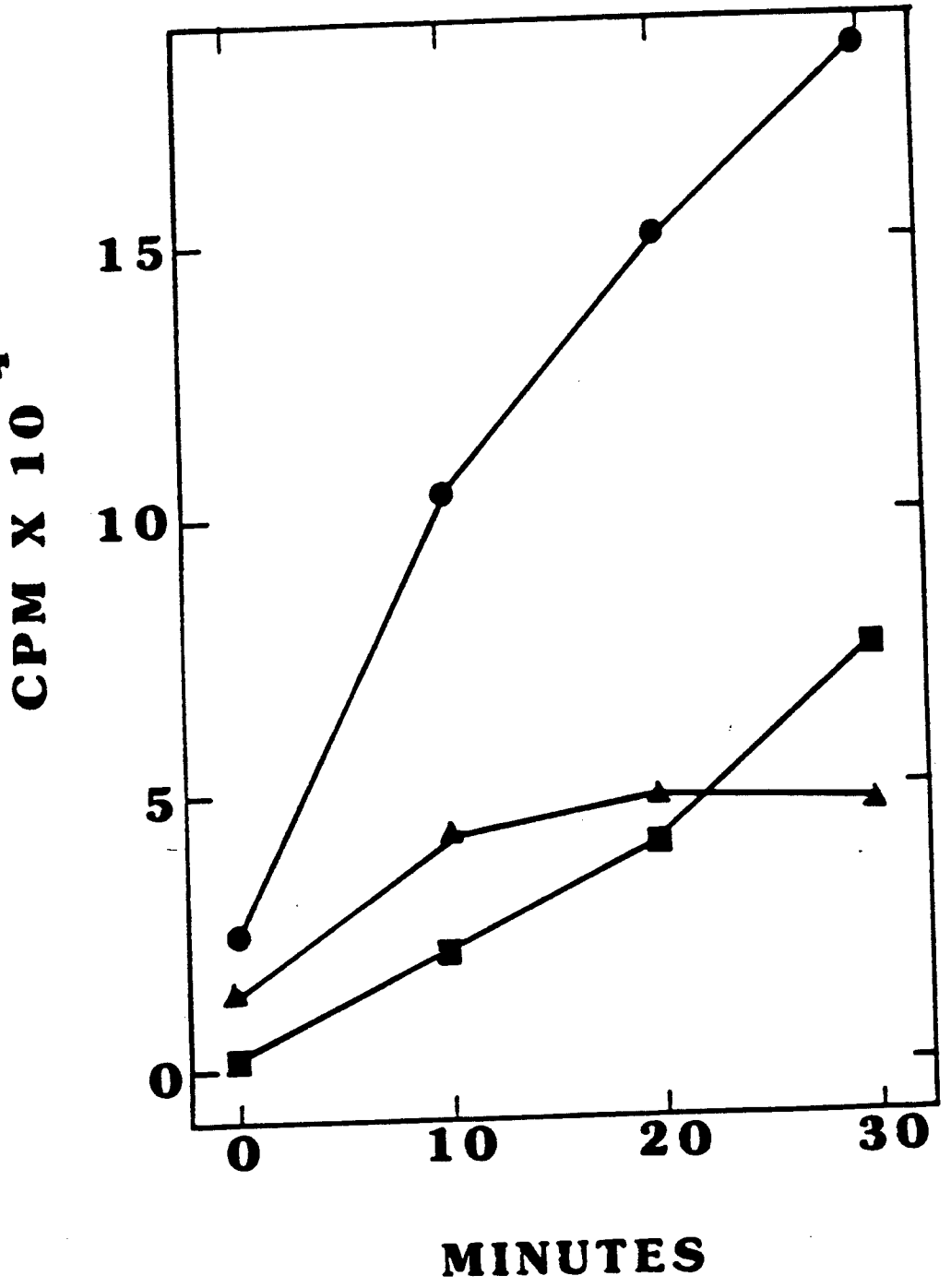
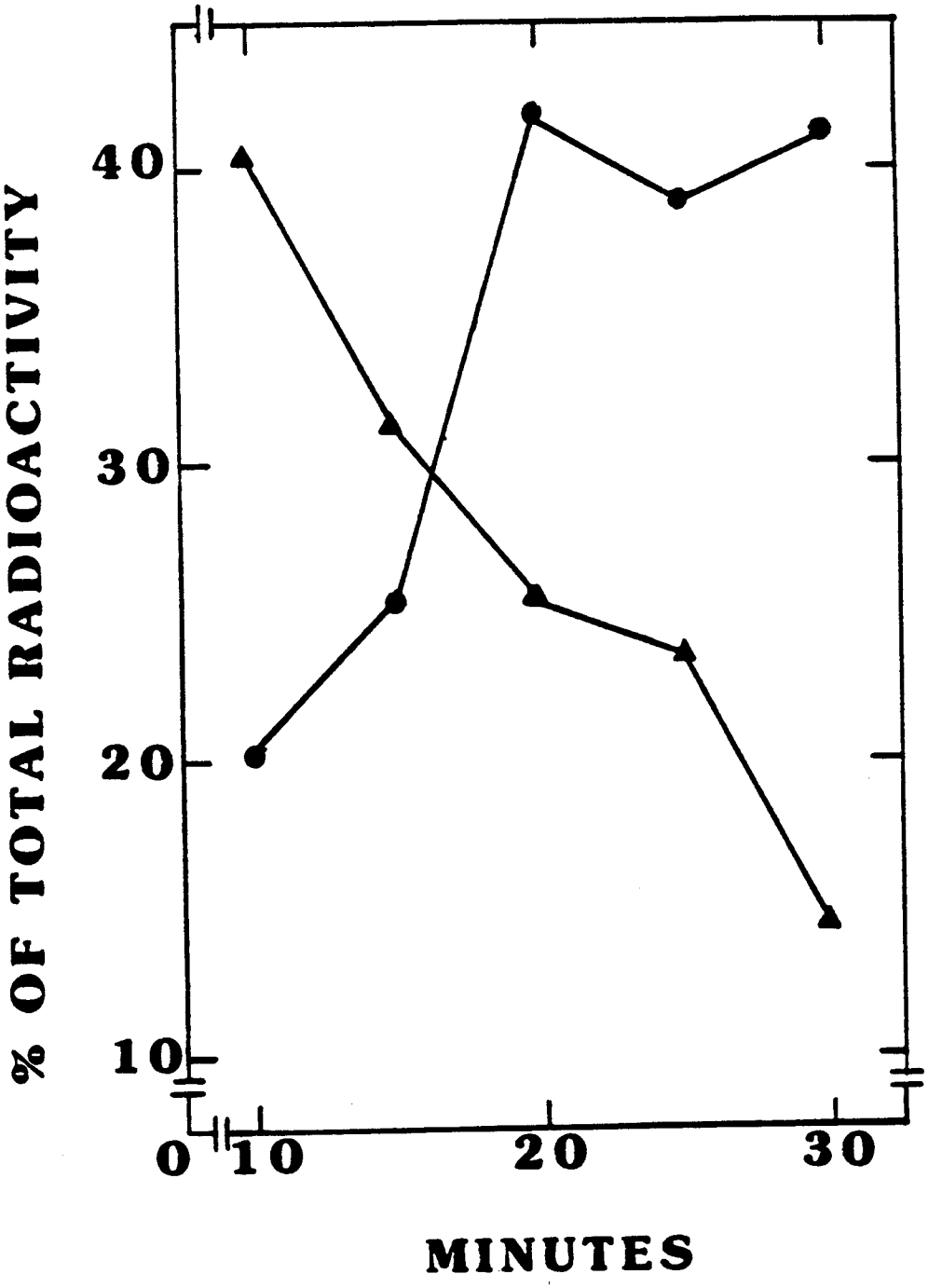

1' (+)
5' (+)
10' (+)
20' (+)
20' (-)
30' (+)
30' (-)
O->
XC->
BPB->
T

Figure 33. Time dependence of the synthesis of poly(ADP-ribose) of different size classes in nucleotide permeable cells. (●——●) Synthesis of total poly(ADP-ribose), (■——■) synthesis of highly complex poly(ADP-ribose), (▲——▲) synthesis of oligomers of ADP-ribose (2 to 17 residues in size).


CPM X 10-4
15
10
5
0
0
10
20
30
MINUTES

increased with time. Figure 34 shows the percentage of total radioactivity present in the gel fragments containing short and complex polymers of ADP-ribose, respectively. It was observed that when radiolabeled poly(ADP-ribose) was chased with cold NAD^+, oligomers of ADP-ribose disappeared while highly complex polymers accumulated. Therefore the small polymers obseved *in vitro* most likely correspond to intermediates in synthesis. Thus, these data support a random mechanism for the generation of points of branching. The probability of branching increases with the polymer size and approaches one at a polymer size of approximately 35 residues.

Figure 34. Percent of total radioactivity in oligo (2 to 17 residues) and highly complex poly(ADP-ribose) synthesized in nucleotide permeable cells in the chase experiment. (●——●) Highly complex polymers. (▲——▲) oligomers of ADP-ribose (2 to 17 residues).


% OF TOTAL RADIOACTIVITY
40
30
20
10
0
10
20
30
MINUTES

CHAPTER V

DISCUSSION

The synthesis and degradation of poly(ADP-ribose) are closely associated with chromatin. Thus, it is reasonable to suggest that the metabolism of the polymer is involved in "chromatin related events". The regulation of chromatin functions by poly(ADP-ribosylation) may be achieved by two general types of mechanism. One of these may be the covalent modification of specific nuclear target proteins. Another mechanism may involve non-covalent interactions between the highly negatively charged polymer of ADP-ribose and other chromatin components.

Other studies have already been aimed at understanding the covalent functions of poly(ADP-ribose). It has been shown that a wide variety of nuclear proteins are poly(ADP-ribosylated) <u>in vitro</u>. These include enzymes such as a Ca^{++}, Mg^{++} endonuclease (183), topoisomerase I (47) and the poly(ADP-ribose) polymerase itself (97,157,181). This event appears to lead to enzyme inactivation. Other covalently modified nuclear proteins include the core histones (141), histone H1 (142) and the high mobility group proteins (141). In fact, it has been proposed that the

histone H1 poly(ADP-ribose) conjugates are involved in the mechanisms of chromatin condensation (154). In contrast, other investigators have suggested that poly(ADP-ribose) might be involved in the mechanisms of chromatin relaxation (19,40). Therefore, more data are required to demonstrate whether or not poly(ADP-ribose) is involved in the mechanism of chromatin condensation or relaxation or both.

Our current understanding of the covalent acceptors of poly(ADP-ribose) chains in vivo is still very limited. For instance, only recently it has been reported that histone H2B and the poly(ADP-ribose) polymerase may be the major protein acceptors for poly(ADP-ribosylation) in intact cells following DNA damage (3,102). Further, Tanuma and Johnson have reported that the high mobility group proteins are also poly(ADP-ribosylated) in vivo (163). Furthermore, It has also been suggested that two histone H1 molecules are crosslinked by poly(ADP-ribose) molecules of 15 or 16 residues in size (178), giving rise to the so-called histone H1 dimer or H1 complex. Evidence that H1-poly(ADP-ribose) conjugates of higher complexity than the histone H1-dimer exist in intact cells has been documented as well (154). It has also been suggested that the activity of DNA ligase II is regulated by poly(ADP-ribosylation) (37). This enzyme activity has been suggested to be responsible for the ligation of DNA nicks in the DNA repair replication mechanisms. However, it was not shown whether poly(ADP-

ribose) was covalently bound to the enzyme. It is clear, that studies aimed at understanding the covalent interactions of poly(ADP-ribose) with chromatin proteins have not as yet conclusively demonstrated not even one of the many possible biological function(s) of the polymer in chromatin.

It is important to emphasize that essentially no attention has been given to the importance of non-covalent interactions of poly(ADP-ribose) with other chromatin components. Information concerning the polymer size and complexity *in vivo* should be an important factor in our understanding of the non-covalent role(s) of poly(ADP-ribose) in chromatin. No information concerning the size and complexity of poly(ADP-ribose) *in vitro* and *in vivo* has thus far appeared in the literature. This is due to the lack of suitable methodology to do such studies. The development of suitable methodology to evaluate the size and complexity of poly(ADP-ribose) must meet several criteria. (1) It is an absolute requirement to isolate poly(ADP-ribose) from other interfering macromolecules of similar structure such as RNA or DNA that exist in the cell in far greater amounts. (2) It is necessary to fractionate polymers on the basis of size using methodology that allows efficient recoveries. (3) A sensitive method for the determination of polymer complexity is required. This also includes an accurate measurement of the frequency of

branching in poly(ADP-ribose) which requires the complete digestion of the polymer to nucleotides or nucleosides and an accurate quantification of the digestion products.

The methodology described in this dissertation meets the above criteria. The purification of poly(ADP-ribose) free of interfering polynucleotides was achieved by the use of a highly selective and specific boronate resin (9). The high dependence of this resin on the ionic strength allowed the efficient elution of bound material with water. The purification of polymers of ADP-ribose synthesized in vitro is relatively easy since radiolabeled NAD^+ can effectively be used to obtain labeled polymers using systems such as permeabilized cells, purified poly(ADP-ribose) polymerase preparations or isolated nuclei. However, the purification of polymers synthesized in vivo is a much more difficult problem because labeled NAD^+ can not be used, since it is not permeable to cells. Thus, an alternative method has been developed to radiolabel the NAD^+ pool to a sufficiently high specific radioactivity in vivo. Johnson and Tanuma (163) have recently described the labeling of poly(ADP-ribose) in vivo with [^{3}H] adenosine. However, the total incorporation of radiolabel in the polymer was less than 0.01%. In the studies presented here, high specific activity [^{3}H] Ade was effectively used to label the Ade nucleotide pools, including NAD^+, in cultured mouse cells. It is extremely important to isolate radiolabeled poly(ADP-

ribose) synthesized in intact cells free of other interfering material that would yield radiolabeled 5' AMP upon phosphodiesterase digestion and thereby cause an underestimation of polymer size. The protocol utilized here combined with the control experiments done make it very unlikely that the polymer size is being underestimated in intact cells. Experiments concerning the alkaline stability of the polymer have failed to detect RNA contamination. Additional results obtained in this work can also rule out serious underestimation of polymer size due to other sources of 5' AMP. For example, possible contamination with alkaline-resistant RNA fragments containing 2,O-methyl ribose is eliminated by the fact the it would not be expected to bind to the boronate resin utilized here, which requires at least two 1,2 cis-diol groups for binding. In addition, contamination of 5' AMP coming from protein bound monomeric ADP-ribose is eliminated by the strong alkaline treatment of the acid insoluble material prior to affinity chromatography (table II). It has been shown that diadenosine tetraphosphate (Ap_4A) is an acceptor for poly(ADP-ribosylation) <u>in vitro</u> in the presence of histones. However, the concentrations of Ap_4A used there were extremely high and the size of the polymers attached to the dinucleotide did not exceed three residues (160). Further, the intracellular levels of Ap_4A in intact cells are extremely low (11). In addition, this dinucleotide is acid

soluble. Thus, any possible contamination of the polymer preparations with Ap_4A is eliminated at the first step of the protocol used for polymer isolation which involves acid precipitation. Even if a small amount of the dinucleotide was trapped during polymer precipitation, it should have been eliminated by molecular sieve chromatography, since it should elute at the position of monomers or dimers of ADP-ribose (figure 12). However, one could argue that the dinucleotide could be attached to the reducing end of poly(ADP-ribose) molecules *in vivo*. Thus, Ap_4A would give an additional 5' AMP residue following enzyme digestion of the polymer to nucleotides. This might cause an underestimation of the polymer size by two fold. This possibility is also ruled out by a direct comparison of the electrophoretic migration of polymer fractions (figure 13) with the polymer size determined by the chemical analysis (table IV). If in fact the polymer size was underestimated, the close correlation between the calculated size and expected electrophoretic migration would not have been observed. Further, it was observed that there was an exellent agreement between corresponding molecular sieve chromatographic fractions of polymers generated *in vitro* (table V) and intact cells (table IX). Such close agreement would not be possible if the preparations labeled *in vivo* were contaminated with other sources of 5' AMP.

The fractionation of poly(ADP-ribose) samples according to size was effectively achieved by two different methodologies, polyacrylamide gel electrophoresis (figures 7 8, 13, 17, 18 and 19) and by HPLC-molecular sieve chromatography (figures 11, 12, 26, 27 and 30). Although the former method gave excellent resolution in the separation of polymers of different size. It was observed that the latter method was more efficient for polymer recovery where routinely more than 95% of the sample was recovered. This was crucial to accurately determine the branching frequency and thus, the polymer complexity. Previously, estimates of the average chain length of polymers generated in vitro have been made using an analytical approach valid for linear polymers but not for branched polymers (4,117,159). In those studies SVPD was used to generate the nucleotides PRAMP from linear internal residues and AMP from terminal residues. At that time the branched structure of poly(ADP-ribose) had not been elucidated (121). Even if it had been known, the precise quantification of branching frequency would have been difficult since the points of branching occur at an average of approximately 1 in 40 residues (121). Thus, the amount of branched residues following SVPD digestion of the polymer does not exceed 3 mole %. The separation of nucleotides by paper chromatography following digestion is not satisfactory for the detection of branching residues. It is important to

mention that SVPD needs to be purified prior to being used for the determination of average polymer size and complexity because commercial preparations are ususally contaminated with 5' nucleotidase. The presence of this enzyme may lead to the underestimation of the nucleotide amounts. In addition, the polymer digestions were performed in the presence of phosphate buffer to inhibit any possible residual phosphatase activity. The nucleotides generated were then separated using a system developed in the laboratory (figure 22). Thus, it was possible to determine the size and complexity of labeled polymers of ADP-ribose synthesized in vitro (table V) and in vivo (tables VII and IX) by this method.

It is important to note that the methodology developed here also allowed the evaluation of polymer complexity under conditions when radiolabeling is not possible (tables X and XI). This was achieved by the fluorescent detection of etheno-nucleosides by HPLC-reversed phase chromatography, (figure 23). However, there is one disadvantage to consider before the analysis of polymer complexity by this method is done. This is related to the contamination of commercial preparations of BAP with polynucleotides that yield Ado, following incubation of polymer samples with SVPD and BAP. This may cause an underestimation of the polymer size. This problem can be resolved by the preincubation of the enzyme mixture followed by dialysis as indicated in methods.

However, it is still necessary to run a control without a sample to quantify the amounts of Ado coming from the enzymes. The use of this method to evaluate the complexity of poly(ADP-ribose) should prove useful in future studies where the use of radiolabeling techniques is not possible.

Evaluation of the size and complexity of [^{32}P] poly(ADP-ribose) synthesized in vitro revealed polymers of a maximum average size of 190 residues that contained as many as 6 points of branching per molecule. It is important to note however, that since only points of branching were determined, it was not possible to derive from these data, information concerning the actual size of the branches in the polymer, i.e. a single residue or elongated branches with multiple residues. It is interesting to note that the branching frequency of poly(ADP-ribose) approaches one at an approximate polymer size of 35 residues. This size corresponds to the molecular sieve chromatography fractions where large amounts of non-migrating polymers are observed on polyacrylamide gels. Therefore, it is tempting to speculate that branches in poly(ADP-ribose) may be responsible for the anomalous electrophoretic migration of these highly complex polymers. It is also interesting to note that the fraction of this material that migrated into 4% polyacrylamide gels did not resolve in bands. This was expected, since this material exclusively contains branched polymers and the number of possible structural isomers of

branched polymers are too numerous to be separated by this method. This anomalous behavior of complex polymers of ADP-ribose on polyacrylamide gels further suggests that the branching points are indeed likely to be substantially longer than a single residue. Electron microscopic observations of multibranched polymers of ADP-ribose synthesized in vitro are also consistent with polymers containing elongated branches, although, it was not clear if the complex structures observed represented single polymer chains (40,64).

In addition, the data presented here concerning polymers generated in vitro suggested that the mechanism of branching in poly(ADP-ribose) is random. This conclusion was made based on the observations that relatively short oligomers of ADP-ribose (under 20 residues in size) contained significant amounts of branching. The polymer synthesized in nucleotide permeable cells is completely stable and little or no turnover takes place, since the system is depleted of poly(ADP-ribose) glycohydrolase activity (figures 31 and 32). Further, these polymers were shown to be intermediates in synthesis and not degradation products of highly complex polymers of ADP-ribose (figures 32 to 34). Thus, poly(ADP-ribose) molecules do not have to reach a given size for poly(ADP-ribose) polymerase to catalyze branching of the polymer. Therefore, the data obtained are consistent with a random mechanism of

branching. The probability of branching in poly(ADP-ribose) increases with polymer size and approaches one at a polymer size of 35 residues.

In order to evaluate the complexity of poly(ADP-ribose) in vivo, the polymer size and branching frequency of poly(ADP-ribose) were determined in intact confluent mouse cells in tissue culture. It is known that poly(ADP-ribose) metabolism is rapidly altered following DNA damage (39,92,-111,177). Studies with inhibitors of ADP-ribosylation reactions argue that these metabolic changes are necessary for chromatin associated events that ultimately lead to cellular recovery from the cytotoxic effects of alkylating agents. In addition, it has recently been reported that hyperthermic exposure modulates poly(ADP-ribose) metabolism (89). This modulation requires protein but apparently not RNA synthesis and involves primarily a decreased turnover of the polymer (83). The activity of poly(ADP-ribose) glycohydrolase appears to be negatively regulated following heat shock. The levels of poly(ADP-ribose) are elevated several hundred fold following exposure to either hyperthermia or alkylating agents. Thus, these conditions were used to stimulate poly(ADP-ribose) synthesis in intact cells. It is important to note that these conditions are only potentially lethal to confluent cells. In contrast, dividing cells are more sensitive to these treatments. Thus, non-dividing cells were used for the studies described

here. The survival rate of non-dividing cells, 48 hours posttreatment with MNNG is about 50%. The survival rate 48 hours posttreatment with hyperthermia followed by DNA damage with MNNG is 25%. Stressful conditions were used to study polymer complexity in intact cells because it has been suggested that changes in the metabolism of poly(ADP-ribose) are required for cell growth and development under these conditions. Inhibitors of poly(ADP-ribose) synthesis have failed to demonstrate that the polymer is required for normal cellular growth and development under basal conditions. It was observed that polymers of considerable size and complexity were synthesized following MNNG treatment. These polymers had an average size of 67 residues and contained at least 2 branching points per molecule. These observations were somewhat surprising because of the relatively fast rate of turnover of poly(ADP-ribose) following DNA damage. The half-life of poly(ADP-ribose) following DNA damage with MNNG is about 1 min (83). Under conditions of hyperthermia and DNA damage even larger and more complex polymers of ADP-ribose were generated. These polymers had an average size of 244 residues and contained at least 6 points of branching per molecule.

The difference in polymer complexity observed between polymers synthesized following DNA damage and the polymers synthesized following a combination of hyperthermia and DNA damage may be due to either one of two reasons. It is

possible that the size and complexity of poly(ADP-ribose) are required to be different depending on the chromatin structural reorganization taking place following either treatment. The other possibility is that more complex polymers are observed following hyperthermia and DNA damage due to the decreased rate of polymer turnover. The rate of turnover of poly(ADP-ribose) is decreased at least 30 fold following heat shock and DNA damage (83). The half-life of poly(ADP-ribose) following hyperthermia and MNNG treatment is more than 30 min (83). Thus, it is reasonable to suggest that it would be difficult to observe highly complex polymers of ADP-ribose following DNA damage because of the rapid turnover of poly(ADP-ribose).

In view of the data presented here, it is possible that the histone H1-poly(ADP-ribose) conjugates observed by other investigators (98,178) may contain highly complex polymers rather than covalent crosslinks. It is interesting to note that the maximum size and complexity of poly(ADP-ribose) in nucleotide permeable cells and in intact cells following heat shock and DNA damage is about the same. In both cases the maximum average polymer size observed was about 200 residues and the number of branching points per molecule was between 6 and 7. It has been proposed that nucleosomes are condensed in higher ordered chromatin structures referred to as solenoids (28). The solenoid is believed to have 6 to 8 nucleosomes per turn (48,148), where

the histone H1 is located at the internucleosomal regions towards the center of the solenoid. The fact that poly(ADP-ribose) molecules with as many as 6 to 7 branches per molecule occur in chromatin may suggest that the formation of crosslinked histone H1 via branched poly(ADP-ribose) either covalently or non-covalently could be responsible for the transient stabilization of solenoid structures.

Since polymers of ADP-ribose carry two formal negative charges per residue and polymers of considerable size and complexity are observed in intact cells under different sets of conditions, the results presented here argue that non-covalent interactions between the polymer and other components of chromatin should receive serious further consideration in future studies of poly(ADP-ribose) metabolism and regulation of chromatin functions.

In addition, the methodology developed here should also prove useful for studies regarding localization of poly(ADP-ribose) within chromatin. The evaluation of the complexity of polymers bound to a given chromatin protein *in vivo* must yield additional information about the covalent functions of poly(ADP-ribose) as well. It is very unlikely that poly(ADP-ribosylation) modification of proteins serve only to regulate enzyme activities important for chromatin to recover following DNA damage or stress.

CHAPTER VI

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