POLY(ADP-RIbose) SYNTHESIS AS A FUNCTION OF GROWTH AND DNA FRAGMENTATION

DISSERTATION

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By

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This work examines the synthesis of poly(ADP-ribose) in normal and SV40-transformed monolayer cultures of 3T3 cells as a function of growth and DNA fragmentation. A review of the relevant literature is given in the introduction of this work. Poly(ADP-ribose) synthesis has been implicated in transcription, replication, repair, differentiation and regulation of cell growth. The results of this study suggest that poly(ADP-ribose) synthesis is involved in some aspect of cell-growth control and DNA repair.

The work presented here mainly examines poly(ADP-ribose) synthesis as a function of growth in permeabilized cells from monolayer cell cultures which exhibit density-dependent inhibition of growth (DDIG) and their transformed counterparts which have lost this property. A six-fold increase in the synthesis of the polymer was observed as normal cells entered DDIG. At corresponding cell densities, this increase in polymer synthesis was not observed with the transformed cells. This observation suggests that poly(ADP-ribose) may be involved in growth regulation.

This is the first study which describes the simultaneous examination of NAD degrading enzymatic activities in permeable 3T3 cells, namely, poly(ADP-ribose) polymerase, NAD
glycohydrolase and NAD pyrophosphatase. The observation that NAD pyrophosphatase is the major NAD degrading enzymatic activity in permeable cells and that it increases threefold as cells enter DDIG, might suggest that this enzyme is also involved in growth control.

This work carefully examines the relationship between poly(ADP-ribose) synthesis and DNA strand breaks. Analysis of the cellular DNA by velocity sedimentation on alkaline sucrose gradients revealed that the increase in polymer synthesis observed in nongrowing cell cultures is not associated with DNA fragmentation.

Another aspect of this work was to investigate the effect of DNA-damaging agents, DNase I and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) on NAD degrading enzymatic activities in permeable 3T3 cells. It was observed that only poly(ADP-ribose) polymerase activity is stimulated by such treatment. This observation is in agreement with other studies which strongly suggest that poly(ADP-ribose) synthesis might be involved in DNA repair processes.
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CHAPTER I

INTRODUCTION

Nicotinamide adenine dinucleotide (NAD) has long been known to be an electron carrier in various biological oxidation-reduction systems. In addition, a number of reactions have been discovered in which NAD serves as a substrate. For example, certain prokaryotes, such as Escherichia coli, utilize NAD as a substrate for deoxyribonucleic acid ligase (105, 141). NAD also serves as a substrate in reactions generally termed ADP-ribosylations. The ADP-ribose moiety of NAD is transferred to an acceptor molecule with the concomitant release of the nicotinamide ring as shown in the equation below:

\[
\text{Acceptor} + \text{NAD} \rightarrow \text{Acceptor-ADP-ribose} + \text{Nicotinamide} + \text{H}^+ 
\]

The reaction is termed "mono ADP-ribosylation" when only a single unit of ADP-ribose is added to an acceptor. The subsequent addition of ADP-ribose units to mono ADP-ribosylated acceptors forms poly(ADP-ribosylated) molecules as shown in the following equation:

\[
\text{Acceptor-ADP-ribose} + n\text{NAD} \rightarrow \text{Acceptor-(ADP-ribose)}_{n+1} + n\text{Nicotinamide} + n\text{H}^+ 
\]
ADP-Ribosylations In Vitro

Diphtheria toxin is an exotoxin produced by strains of *C. diphtheriae* that are infected with β-phage. This toxin inhibits protein synthesis in susceptible organisms without inhibiting RNA synthesis, glycolysis or respiration (197). Kato and Pappenheimer reported that the inhibition of protein synthesis required NAD (99) and resulted from the inactivation by the toxin of EF-2, an enzyme which participates in the elongation of polypeptide chains in eukaryotic cells (37,38,69). Honjo et al. (81-83) demonstrated in 1968 that inactivation of EF-2 by diphtheria toxin involved ADP-ribosylation. Recent reports show that *Pseudomonas* toxin catalyzes the same reaction, inactivating EF-2 (85). Cholera toxin catalyzes the ADP-ribosylation of the regulatory subunit of adenylate cyclase resulting in an increase in the levels of cAMP (65). Recent reports indicate that turkey erythrocytes contain an ADP-ribosyltransferase which activates adenylate cyclase (126). The DNA-dependent RNA polymerase of *E. coli* is ADP-ribosylated following infection with bacteriophage T4 (67, 157). Infection by the coliphage N4 causes ADP-ribosylation of a variety of host specific proteins, but unlike the T4 enzyme, it does not modify RNA polymerase (145). Roberts et al. (156) have reported an ADP-ribose polymerizing activity associated with ribosomes in HeLa cells which closely resembles nuclear poly(ADP-ribose) polymerase in its requirements.
Kun et al. (103) have reported the existence of an enzymatic system in rat liver mitochondria which transfers the ADP-ribose moiety of NAD to a protein with a molecular weight of 100,000 daltons. This reaction was reversible and a fraction of the protein-bound ADP-ribose appeared to be present as an oligomer.

Formation of poly(ADP-ribose) in vitro was first demonstrated in liver nuclei. The enzymatic system located in chicken liver nuclei incorporated (alpha-\(^{32}\)P)ATP into an acid-insoluble product when supplemented with NMN (32, 33). Incubation of (\(^{32}\)PNMN and ATP, (ribose-\(^{14}\)C) NMN and ATP, or (\(^{32}\)P) NAD with chicken liver nuclei resulted in an acid-insoluble labeled product. However, incubation of nuclei with (nicotinamide-\(^{14}\)C) NMN and ATP or with (nicotinamide-\(^{14}\)C)NAD did not form a labeled material, suggesting that the polymer consisted of ADP-ribose moieties derived from NAD and it was formed by the polymerization of the ADP-ribose units. Hydrolysis of the polymer by snake venom phosphodiesterase produced a nucleotide containing two ribose and two phosphate residues per adenine, and the phosphate groups were removable by treatment with alkaline phosphomonoesterase. Hydrolysis of this nucleotide by dilute mineral acid liberated ribose-5-phosphate and 5'-AMP. Consumption of one mole of periodate per mole of this nucleotide showed that only one of the two riboses had free adjacent hydroxyl groups at the 2' and 3' positions,
indicating a glycosidic linkage between ribose-5-phosphate and the 2' or 3' of 5'-AMP (32,33,76,151). Methylation of the dephosphorylated compound followed by acid hydrolysis yielded N\(^6\)-methyladenine, 2,3,5-trimethylribose and 3,5-dimethylribose, indicating an O-glycosidic linkage between 1' and 2' carbons of the two riboses. The proposed structure is shown in Fig. 1. Incubation of radioactive 2'-deoxy-NAD with rat liver and HeLa nuclei resulted in the formation of a covalent linkage with nuclear proteins. Snake venom phosphodiesterase treatment of the product liberated phosphoribosyl-2'-dAMP, which has been characterized and shown to have a 1'→3' glycosidic linkage, suggesting that the polymerizing enzyme does not require a 2'-hydroxyl group for glycosidic bond formation (184). Recently, using carbon thirteen nuclear magnetic resonance, the configuration of the O-glycosidic linkage between the ADP-ribose monomers has been established to be an alpha linkage (54,87).

A method for the determination of the average chain length of the polymer was established by early investigators (151). When the polymer is digested with snake venom phosphodiesterase, the pyrophosphate linkage is hydrolyzed to yield one molecule of 5'-AMP from the distal terminus, a number of 2'-(5''-phosphoribosyl)-5'-AMP (PR-AMP) residues, and one molecule of ribose-5-phosphate attached to an acceptor molecule. Therefore, the number of AMP molecules
Figure 1

Structure of Poly(ADP-ribose).
represents the number of polymer chains. The average chain length can be calculated by dividing the total number of the AMP and PR-AMP residues by AMP residues as shown in the following equation:

\[
\text{Average Chain Length: } \frac{(5'-AMP + PR-AMP)}{(5'-AMP)}
\]

The chain length of poly(ADP-ribose) as determined by this method ranges from 1 to about 39 monomer units, depending on the experimental conditions. A rapid method for purifying poly(ADP-ribose) after incubation of nuclei preparations with labeled precursors was reported by Sugimura et al. (182). The crude preparation was digested with pronase to remove proteins, extracted with phenol, and applied to a hydroxyapatite column. Elution was carried out with increasing concentrations of phosphate buffer which separated RNA and DNA from poly(ADP-ribose). This technique has also been used to examine the size distribution of the poly(ADP-ribose) chains (51,187). More recently, Tanaka et al. (188) have utilized high resolution poly-acrylamide gel electrophoresis to examine the size distribution of the poly(ADP-ribose) chains. When examined by this method, the polymer was found to have chains up to 65 residues long. However, the chain length of each fraction, estimated as the ratio of total residues of ADP-ribose to the number of termini after complete hydrolysis with snake
venom phosphodiesterase, never exceeded 30. This finding was explainable only if poly(ADP-ribose) had a branched structure. Miwa et al. (121) have recently isolated a small component from (ade-^{14}C) poly(ADP-ribose) digested with snake venom phosphodiesterase which contained 2% of the total radioactivity applied to a column of DEAE-Sephadex A-25. This nucleotide had an adenine/ribose/phosphate ratio of 1/2.7/2.8. The structure was determined to be 2'-(1'-ribosyl'2''-(or 3'') (l''-ribosyl) ) adenosine-5'-5''-5''' triphosphate, upon mass spectroscopic analysis of the permethylated phosphorylated compound. Miwa et al. (122) have recently reported that the O-glycosidic linkage of the proposed branch nucleotide is alpha 2'->l''' as shown in Figure 2.

The first reports by Nishizuka et al. (132,134) on ADP-ribosylated proteins indicated that a large percentage of (ADP-ribose) was associated with the histone fraction. When ribose-labeled NAD was incubated with rat liver nuclei and the reaction product was analyzed, most of the acid-insoluble label was extracted with 0.25 N HCl, and upon carboxymethylcellulose column chromatography, the radioactivity coincided exactly with the location of carrier histones, H1, H2A, H2B, and H3. Tanuma et al. (189) have isolated histones from HeLa cell nuclei by extraction with 0.4N H_{2}SO_{4}. Electrophoresis on polyacrylamide gels revealed that 85% of the poly(ADP-ribose) was associated with the
Figure 2

Structure of the proposed branched nucleotide of poly(ADP-ribose).
histone H1 fraction. Stone et al. (178) have reported the formation in HeLa cell nuclei of a complex of two histone H1 molecules held together by covalent association with a 15-residue-long poly(ADP-ribose) chain. This represented 6% of the polymer synthesized in HeLa nuclei. Adamietz et al. (3), however, were unable to detect the histone H1 dimer and reported that H1 contained less than 1% of the ADP-ribose residues synthesized in HeLa nuclei. Giri et al. (66) reported that histones H1 and H2B were major acceptors of poly(ADP-ribose) in HeLa nuclei, whereas H2A and H3 were modified to a lesser extent. Several non-histone proteins have been reported to be modified by ADP-ribosylations. Yoshihara et al. (206) have reported the ADP-ribosylation of Ca$^{++}$, Mg$^{++}$-dependent endonuclease with concomitant inhibition of the enzyme activity. Activation of an acid endonuclease by ADP-ribosylation has also been reported (137). Yoshihara et al. (207) reported that poly(ADP-ribose) polymerase purified from calf thymus catalyzed self poly(ADP-ribosylation). Okayama and Hayashi (139) have reported that the nuclear protein A24, which is composed of a histone H2A molecule joined by an isopeptide linkage to ubiquitin (68), is ADP-ribosylated in rat liver nuclei. Giri et al. (66) have also reported that the high mobility group (HMG) proteins and a group of proteins designated M1-M4 are ADP-ribosylated. In rapidly growing
tissues, most ADP-ribose residues were associated with the non-histone fraction (25,45).

The existence of poly(ADP-ribose) in the form of covalently linked poly(ADP-ribose) proteins was postulated first by Nishizuka et al. (134,135) on the basis of ADP-ribose residues which could be detached from protein by treatment with neutral NH$_2$OH or dilute alkali. Based on this observation, it was proposed that poly(ADP-ribose) was linked to protein by an ester bond. Covalent binding of ADP-ribose residues to nuclear proteins was also observed in the experiments of Otake et al. (143), who performed CsCl centrifugation in the presence of guanidinium chloride. Experiments revealed that short as well as long (ADP-ribose) chains are linked to proteins by two different type of bonds, one being susceptible to alkali and NH$_2$OH, the other alkali-labile but NH$_2$OH resistant (1,2). Dixon et al (46) reported evidence supporting an ester linkage via glutamic acid residues in histone H1. Further evidence for the modification of glutamic acid residues in histone H1 and histone H2B came from the work of Burzio et al. (27), Riquelme et al. (153) and Ogata et al. (136). Smith and Stocken (168,169) have reported that poly(ADP-ribose) is linked via a serine phosphate to histone H1.

The enzyme responsible for the synthesis of poly(ADP-ribose) was first observed in the particulate fraction of nuclear extracts from hen liver (33) and from rat liver.
Enzyme activity was also detected in nuclei of rat testes, brain, kidney, spleen (132); mouse LS cells (162); L5/78Y lymphoma cells (104); Ehrlich ascites carcinoma cells (79); pig lymphocytes (106) and aortic tissue (93); HeLa cells (147,170); leukocytes, chronic lymphatic leukocytes, acute myelogenous leukocytes (25); quail oviduct (127); trout liver (183); carp liver (183); and Physarum polycephalum (22). The enzyme has been purified by Doly et al. (48) 20-fold, by Yamada et al. (202) 10-fold, by Yoshihara (205) 130-fold, by Ueda et al. (198) 5,500-fold, by Mandel et al. (110) 3,000-fold, and by Kristensen and associates (84,102) 689-fold. The enzyme has been reported to have a molecular weight of 78,000 g/mole in rat liver (202), 120,000 daltons in calf thymus (110), 130,000 g/mole for a subunit weight in Ehrlich ascites cells (84,102), and 63,500 g/mole in pig thymus (194). The affinity for the substrate NAD seems to be different for enzymes from different sources. For the enzyme from Ehrlich ascites cells (158), hen liver (48), P. polycephalum (162) and rat liver (63,130), Km values between 240 and 270 μM have been reported. Other investigators have reported Km values for the rat liver enzyme between 800 μM (34) and 70 μM (77). A Km of 1.5 mM has also been reported for the enzyme from LS cells by Stone and Shall (174). The enzyme is inhibited by NAD analogs (147), nicotinamide and nicotinamide analogs (34,147,163), and thymidine and thymidine analogs (147,163). The
enzymatic activity is stimulated two- to four-fold in the presence of histones (202).

Evidence supporting the localization of poly(ADP-ribose) polymerase activity in internucleosomal linker regions of HeLa cell chromatin came from the work of Giri et al. (66). The evidence was based on the observation that dimer, trimer, and oligomer nucleosome fragments, obtained after micrococcal nuclease digestion of chromatin, were active, but mononucleosomes containing linker regions showed very little activity and removal of the linker region resulted in the loss of polymerase activity. Later, Butt et al. (29) reported that the specific activity of the enzyme increases progressively with increasing nucleosome repeat size up to 8 units.

There are two enzymes which are known to degrade poly-(ADP-ribose): poly(ADP-ribose) glycohydrolase and poly(ADP-ribose) phosphodiesterase. The first enzyme splits the ribose-ribose linkage of the polymer to produce ADP-ribose, while the second one cleaves the pyrophosphate bonds to produce 5'-AMP and ribose-5-phosphate from the termini, and PR-AMP from the interior.

The glycohydrolase has been purified from rat liver nuclei (195), calf thymus (119), P. polycephalum (186), and rat testis (26). It is inhibited by ADP-ribose and cAMP (195). The glycohydrolase degrades poly(ADP-ribose) in an exoglycosidic (26,119,186) as well as endoglycosidic (100,
123) fashion. This enzyme is believed to play a major role in vivo in the degradation of poly(ADP-ribose) (124).

The second type of poly(ADP-ribose) degrading enzyme, phosphodiesterase, has been purified from snake venom (58, 151), rat liver nuclei (60), and rat liver mitochondria (52). Futai and Mizuno (60) were the first investigators to discover phosphodiesterase activity in rat liver nuclei. This enzyme is also capable of cleaving the pyrophosphate bonds in NAD, NADH and ADP-ribose (61). Hydrolysis of poly(ADP-ribose) by rat liver phosphodiesterase proceeds in an exonucleolytic manner from the AMP terminus, producing PR-AMP (61). Another phosphodiesterase, which degrades poly(ADP-ribose) internally, has been isolated and characterized in rat liver mitochondria by Ferro and Kun (52). Phosphodiesterase activity was also found in cultured cells of Nicotina tabacum (164), which produces PR-AMP as the reaction product.

Recently, Okayama et al. (140) reported the existence of a novel enzyme in partially purified rat liver cytosol which catalyzes the splitting of a linkage between ADP-ribose and the protein portion of mono(ADP-ribosylated) histone H2B.

**ADP-ribosylations In vivo**

In 1967, Doly and Mandel (47) were the first investigators to report the existence of poly(ADP-ribose) in vivo.
They isolated $^{32}$P PR-AMP from venom phosphodiesterase digests of chicken liver after injection of $^{32}$P orthophosphate into animals. Ueda et al. (196) have isolated a histone fraction with 0.25 N HCl from rat liver nuclei after intraperitoneal injection of $^{14}$C ribose and $^{3}$H adenine. Dietrich et al. (44) and Smith and Stocken (168, 169) isolated ADP-ribosylated histone H1 from rat liver after $^{32}$P orthophosphate administration. Kidwell and coworkers reported the existence of poly(ADP-ribose) in vivo in cultured mouse L cells (39) based on $^{3}$H adenosine labeling and in HeLa cells (159) on the basis of a radio-immune assay. An isotope dilution method was utilized by Stone et al. (177) to detect poly(ADP-ribose) in adult and neonatal rat liver and in Zajdela hepatoma cells. Sakura et al. (159) reported the natural occurrence of poly(ADP-ribose) in calf liver, thymus, brain, kidney, pancreas and spleen by a radioimmune assay. Ferro et al. (55) quantitatively determined poly(ADP-ribose) in pigeon and rat tissues by a radioimmune assay which allowed accurate determination of oligomers above 4 ADP-ribose units long. Niedergang et al. (131) have developed a chemical method for the quantification of poly(ADP-ribose) in pmole quantities. This method uses isolated poly(ADP-ribose) with the subsequent quantitation of one of its components, ADP-ribose or PR-AMP, by the formation of a fluorescent adduct. Bredehorst et al. (21) quantified the protein-bound ADP-ribose residues in
crude tissue extracts by selective alkaline conversion of ADP-ribose to 5'-'AMP and radioimmune assay of 5'-'AMP in the range of 1-40 pmoles. More recently, Sims et al. (165) have described a highly sensitive and selective chemical assay for poly(ADP-ribose) where pmole amounts were quantified by detection of a fluorescent derivative of the digestion product of the polymer, etenoribosyladenosine. This technique has been used for the quantification of poly(ADP-ribose) levels in carcinogen-treated 3T3 cells (95).

Other Enzymes Which Utilize NAD

There are two types of enzymes which cleave the substrate NAD: one which attacks the pyrophosphate bond, the other which hydrolyzes the glycosylic bond linking the ADP-ribose residue to nicotinamide. The former enzymes are called NAD pyrophosphatases. The latter enzymes have long been called NAD glycohydrolases of NADases, although a better name would be NAD glycosylases. Poly(ADP-ribose) polymerase and ADP-ribose transferase, which catalyzes the mono(ADP-ribosylation) reactions, can be considered one type of NAD glycohydrolase in this regard. NAD glycohydrolases utilize water as the acceptor molecule and form acid-soluble ADP-ribose, whereas poly(ADP-ribosylation) and mono(ADP-ribosylation) reactions which utilize proteins as acceptors result in the formation of acid-insoluble ADP-ribose.
NAD Glycohydrolases

NAD glycohydrolases are widely distributed in various microorganisms (43,57,73,96,97) and animal tissues (70,130, 175,185). The enzymes of mammalian origin have been described as being associated, in a variety of tissues from different species, with several subcellular fractions, e.g. endoplasmic reticulum and plasma membranes (5,19,128), nuclear envelope (59,71,197) and lysosomes (115). In mammalian tissues, 80 to 90% of the total cellular NAD glycohydrolase activity is located in the microsomal fraction (88), with about 10% being located in purified nuclei (88,130). Nishizuka et al. (113) suggested the existence of three different NAD glycohydrolases in rat liver: one cytoplasmic and two nuclear enzymes. They reported that only one of the two nuclear enzymes could form poly(ADP-ribose). The \( K_m \) values for NAD for these enzymes were 170 \( \mu M \), 250 \( \mu M \) and 250 \( \mu M \), respectively. Nakazawa et al. (130) noted the resemblance of the rat liver nuclear enzyme forming poly(ADP-ribose) to nuclear glycohydrolase. The nuclear glycohydrolase had a higher pH optimum and a higher \( K_m \) value for NAD than that of the microsomal glycohydrolase. It did not react with NADP and was sensitive to DNase I. Bock et al. (18) have described the existence of a nuclear and microsomal NAD glycohydrolase in Ehrlich ascites tumor cells. The nuclear enzyme was very specific for NAD and was inhibited by nicotinamide,
whereas, the microsomal enzyme could hydrolyze NAD, NADP, and NMN. Romer et al. (158) also found two types of NADases in Ehrlich ascites tumor cells; one in the cytoplasm, one in the nuclei. The optimum pH values of the microsomal and nuclear enzymes were 6.2 and 8.2, respectively. In nuclei, they found a stoichiometric relationship between NAD disappearance, \((\text{ade}^{-14}\text{C})\text{NAD}\) incorporation into acid-insoluble material, and nicotinamide release. This was in contrast to the nuclear preparations of rat liver, where degradation of NAD was greater than the formation of poly(ADP-ribose) (130). Stone et al. (175) have reported that in LS cell nuclei, upon inhibition of poly(ADP-ribose) polymerase by thymidine, no glycohydrolase activity was detected. Green and Dobrjansky (70) have demonstrated the existence of an NAD glycohydrolase in Ehrlich ascites tumor cell nuclei which was not identical with the cytoplasmic NADase or poly(ADP-ribose) polymerase activity. This enzyme had a lower pH optimum and was associated with the nuclear membrane. Ueda et al. (197) have separated a nuclear NADase from poly(ADP-ribose) polymerase by fractionation of rat liver chromatin on Sephadex G-200 columns. This enzyme was DNase-insensitive, heat stable, hydrolyzed NADP as well as NAD, had a pH optimum of 6.5 to 7, and was not inhibited by nicotinamide, thymidine, cAMP or ADP-ribose. The \(K_m\) for NAD was 28 \(\mu\text{M}\). It also lacked transglycosidase activity.
NAD Pyrophosphatases

NAD pyrophosphatases catalyze the cleavage of the pyrophosphate linkage of NAD to produce nicotinamide mononucleotide and adenosine-5' phosphate. This enzyme activity has been found in a number of microorganisms (40, 56, 57, 62, 86, 98) and animal tissues (60, 89, 90). Mammalian NAD pyrophosphatases are mostly particulate and have been reported to be associated with nuclei (161), mitochondria (52, 111, 204), endoplasmic reticulum and plasma membranes (6, 16, 17, 166). There appear to be several different particulate NAD pyrophosphatases. Schlieselfeld et al. (161) reported that the nucleotide pyrophosphatase in rat liver nuclei catalyzed the cleavage of NAD, NADP, and UDP-glucuronic acid as well as UDP-glucose; it was inhibited by nicotinamide, and had a pH optimum of 8.0 to 8.3. Futai and Mizuno (60) reported that the pyrophosphatase isolated from mitochondrial fraction of rat liver was not affected by nicotinamide, had a pH optimum of 10.5 for NAD and had a broad substrate specificity. A pyrophosphatase specific for reduced NAD and NADP has been found by Matsuda and Katsunuma (111) in rat liver mitochondria and microsomes. Bischoff et al. (16) have purified pyrophosphatases from rat liver plasma membranes and from endoplasmic reticulum. Both nucleotide pyrophosphatases were shown to have a molecular weight of 137,000 and to contain carbohydrate moieties. They hydrolyzed a
variety of purine and pyrimidine nucleotides, yielding a 5'-nucleoside monophosphate. Proof for the bimodal distribution of nucleotide pyrophosphatase and for the sidedness of the enzyme of plasma membranes was obtained by enzymic iodination of intact rat liver cells (17). The radiolabel incorporated into the enzyme from plasma membranes was several times higher than that of the endoplasmic reticulum, indicating the accessibility of the plasma membrane enzyme from the extracellular space. Yamaguchi et al. (204) have reported that the main enzymatic cleavage reaction of NAD in the crayfish hepatopancreas is catalyzed by NAD pyrophosphatase which is located in the particulate fraction and is inhibited by AMP and GMP. The partially purified enzyme was localized mainly in the mitochondria and microsomes. Using this preparation, the $K_m$ value for NAD was determined to be 1 mM. NADH was about 3 times better substrate for this enzyme than NAD$^+$. This enzyme did not cleave ATP or NADP. Soluble NAD pyrophosphatases have been demonstrated in pigeon and rabbit liver (89,90). Two different NAD pyrophosphatase activities have been extracted from the soluble constituents of pigeon and rabbit liver. One enzyme splits only reduced NAD and NADP, and the other enzyme cleaves both the oxidized and reduced coenzymes.
Possible Biological Functions

Burzio and Koide, in 1970, were the first to suggest the possible involvement of poly(ADP-ribose) in DNA synthesis (23). They showed that preincubation of rat liver nuclei or chromatin with NAD suppressed the incorporation of \((^3H)TTP\) into DNA. Nicotinamide added to the preincubation medium blocked poly(ADP-ribose) synthesis and prevented the inhibition of DNA synthesis induced by NAD. In contrast, Roberts et al. (155) reported that ADP-ribosylation of nuclear proteins by poly(ADP-ribose) polymerase enhances template-primer activity of HeLa cell nuclear DNA. However, Hilz and Kittler (79) reported that there was no correlation between poly(ADP-ribose) polymerase activity in isolated rat liver nuclei and the rate of DNA synthesis in whole cells, and concluded that poly(ADP-ribose) is not involved in the regulation of DNA synthesis. A number of studies have indicated that poly(ADP-ribose) polymerase activity is higher in the nuclei of dividing cells versus resting cells, regenerating versus resting liver (107), mitogen-stimulated versus unstimulated lymphocytes (106), leukemic versus normal white blood cells (25), hormone stimulated versus unstimulated oviducts (127), and SV40-transformed versus untransformed cells (120).

Many investigators have observed a marked fluctuation of poly(ADP-ribose) polymerase activity during the cell cycle of synchronized cell populations. Smulson et al.
reported that poly(ADP-ribose) polymerase activity was the highest when assayed in HeLa cell nuclei during G1 phase of the cell cycle. In contrast, Colyer et al. (39) reported that in synchronized mouse L-cells the synthesis of the polymer in vivo occurs mainly in the S-phase of the cell cycle. Kidwell and Mage (101), using an antibody to poly(ADP-ribose), observed that poly(ADP-ribose) levels in vivo are highest in HeLa cells at the S-G2 border of the cell cycle and are lowest in G2. Berger et al. (10) studied the relation of poly(ADP-ribose) synthesis to DNA synthesis in Chinese hamster ovary cells synchronized by mitotic selection. They reported that when measured in permeable cells, the synthesis of poly-(ADP-ribose) was elevated during G1, fell to its lowest level during S phase, then increased during G2 and rose to its highest level during G1. Roberts et al. (156) observed that the specific activity of poly(ADP-ribose) polymerase in the cytoplasm of HeLa cells was highest in the S phase and paralleled the rate of DNA synthesis during the cell cycle. Tanuma et al. (190) have developed three different assay systems using intact nuclei, disrupted nuclei, and crude poly(ADP-ribose) polymerase for measuring the level of poly(ADP-ribosylation) during the cell cycle of synchronously growing HeLa S3 cells. With intact nuclei, they observed the highest level of poly(ADP-ribose) synthesis during the M phase; with disrupted nuclei, during mid S-G2
phase; and with the crude enzyme preparation, during the G2 phase.

During the growth cycle of cell cultures, a different response of poly(ADP-ribose) polymerase was observed. The specific activity of the polymerase in isolated nuclei of mouse fibroblast cells (LS cells) grown in suspension cultures was low during log phase, compared to three-fold higher activity during late log phase (176). A similar growth related increase in enzyme activity has been reported by Berger et al. (11) in permeable mouse L cells grown in suspension. Berger et al. (12) also reported that in Chinese hamster ovary cells, poly(ADP-ribose) polymerase activity increases as cells enter stationary phase of growth. In suspension-grown HeLa cell cultures, increased specific activities of poly(ADP-ribose) polymerase were observed at a transition from log to stationary phase, while at stationary phase, enzyme activities were low (172). Poly(ADP-ribose) synthesis in the nuclei of monolayer cultures of normal and SV40-transformed African green monkey kidney and mouse fibroblasts have been examined by Miwa et al. (120) as a function of growth. They reported that the enzyme activity in the nuclei of untransformed cells was low and did not change during the growth phase. On the other hand, the enzyme activity in the nuclei of transformed cells increased with increasing cell density, and it was two- to ten-fold higher than the enzyme activity associated with
the chromatin of untransformed cells.

In 1975, Miller was the first to suggest that poly(ADP-ribose) polymerase may have a functional role in the process of DNA repair (116). He reported that the addition of DNase I to the reaction mixture stimulated the synthesis of poly(ADP-ribose) 4-6 fold, and the main effect of DNase I on the synthesis of the polymer was through the initiation of new chains of poly(ADP-ribose) (117). Whish et al. (199) reported that streptozotocin, a carcinogenic autitumor-antibiotic which lowers intracellular NAD levels, stimulated poly(ADP-ribose) synthesis by 200% in P. polycephalum. Davies et al. (42) suggested the involvement of poly(ADP-ribose) polymerase in the recovery of cells from damage, based on the observation that treatment of L1210 cells with the alkylating agent N-methyl-N-nitrosourea (MNU) in the presence of the polymerase inhibitor, 5'-methylnicotinamide, decreased cell survival considerably. Smulson et al. (171) reported that MNU causes alkali-labile damage to HeLa DNA both in vitro and in vivo. They concluded that NAD promotes DNA repair through the synthesis of poly(ADP-ribose).

Jacobson and Narisimhan (91) reported that NAD-depleted cells are unable to do unscheduled DNA synthesis (UDS), whereas cells with normal levels of NAD are UDS proficient. Jacobson et al. (92) examined the mechanism of NAD lowering in N-methyl-N'-nitro-N-nitrosoguanidine (MNNG)-treated 3T3 cells. They reported that in permeable 3T3 cells,
MNNG does not alter NAD glycohydrolase activity but stimulates poly(ADP-ribose) polymerase activity seven-fold. They also reported a rapid time- and dose dependent lowering in the intracellular pools of NAD with MNNG, with no effect on the rate of biosynthesis of NAD in the presence of the carcinogen. Juarez-Salinas et al. (95) have reported that in vivo treatment of SV40 virus-transformed 3T3 cells with MNNG produces, concomitantly with a decrease in NAD levels, a dramatic increase in the intracellular levels of poly(ADP-ribose). Durkacz et al. (49) reported that treatment of L1210 cells with dimethyl sulphate (DMS) lowered the intracellular NAD levels in a dose-dependent manner. This decrease in NAD content was accompanied by a several fold increase in poly(ADP-ribose) polymerase activity. The decrease in NAD levels induced by DMS was prevented by the presence of the inhibitors of poly(ADP-ribose) polymerase. Inhibitors of poly(ADP-ribose) polymerase also prevented the rejoining of DNA strand breaks caused by DMS, as shown by analysis on alkaline sucrose gradients. They also reported that the rejoining of strand breaks was inhibited in NAD-depleted cells.

Berger et al. (13) reported that human lymphoblasts from xeroderma pigmentosum (XP) patients which can repair alkylation damage such as that caused by MNNG show increased poly(ADP-ribose) polymerase activity in permeable cells pretreated with the carcinogen. However, UV irradiation of the same cells does not cause a stimulation in
polymerase activity, since these cells are deficient in
the repair of UV damage. McCurry and Jacobson (114) have
studied the effect of UV light or MNNG on the NAD levels
of human fibroblasts which were homozygous or heterozygous
for the XP genotype. They reported that all cell lines had
reduced levels of NAD following MNNG treatment. Treatment
of normal human fibroblasts with UV light also caused a
rapid time- and dose-dependent lowering of cellular NAD
levels which could be blocked by theophylline, which is a
potent inhibitor of poly(ADP-ribose) polymerase. Following
UV treatment, the NAD content of XP cells was unchanged.
However, XP cells with the variant genotype, which have
normal excision repair, showed a larger reduction in the
size of the NAD pool after UV treatment. Cell lines which
were heterozygous for XP genotype showed an amount of low-
ering of NAD following UV treatment that was one-half that
of the control cell lines. Based on these results, they
concluded that a relationship exists between the conversion
of NAD to poly(ADP-ribose) and DNA excision repair in human
cells. Benjamin and Gill (7) reported that HeLa cell
ghosts rapidly lowered their NAD levels when exposed to
X-irradiation and resulted in the synthesis of poly(ADP-
ribose) chains of high molecular weight. They also showed
that the ability of DNA to support poly(ADP-ribose) synthe-
sis is dependent upon the number and type of strand breaks
it contains and is independent on the sequence (3).
In 1969, Haines et al. (74) reported the possible involvement of poly(ADP-ribose) in RNA synthesis, based on the observation that differentiated liver cell nuclei engaged in RNA synthesis exhibited a higher poly(ADP-ribose) polymerase activity. Other reports in the literature, however, demonstrated that in liver nuclei, the formation of poly(ADP-ribose) did not alter the capacity to synthesize RNA (24,154). The association of poly(ADP-ribose) polymerase with transcriptionally active HeLa chromatin has been reported by Mullins et al. (129). In contrast, Yukioka et al. (209) concluded that the polymerase was not preferentially associated with the transcriptionally active rat liver chromatin, but equally distributed between transcriptionally active and inactive fractions.

Caplan and Rosenberg (31) have reported that differentiation of embryonic chick limb cells into chondrogenic cells in vitro was associated with an increased rate of poly(ADP-ribose) synthesis in vitro, and that addition of nicotinamide to the culture medium inhibited the expression of differentiated state. Yamada et al. (203) reported that inclusion of poly(ADP-ribose) in the culture medium induced the differentiation of murine myeloid leukemia cells. This differentiation was characterized by increased phagocytic activity, increased lysozyme activity, and appearance of Fc receptors. Terada et al. (192) reported
that compounds that were strong inhibitors of poly(ADP-ribose) polymerase in vitro induced differentiation of murine erythroleukemia cells (MELC) in culture. This inhibitory effect on the enzyme activity was not an essential property of inducers of differentiation; N'-methylnicotinamide, the most potent inducer of differentiation, did not inhibit poly(ADP-ribose) polymerase in vitro. Zlatanova and Swetly (211) have reported that poly(ADP-ribose) polymerase activity increased three-fold during the induced erythropoietic differentiation of Friend cells, and that the increased enzymatic activity involved mainly the ADP-ribosylation of the histones.
CHAPTER II

MATERIALS

Balb/c 3T3 mouse embryo fibroblasts and SV40-transformed Balb/c 3T3 mouse fibroblasts (SVT2) were initially obtained from Dr. George Todaro, National Cancer Institute (Bethesda, MD). Saccharomyces cerevisiae strain DK-8 was kindly provided by Dr. David Kaback, Rosensteil Basic Medical Sciences Research Center, Brandeis University. $(^{3}H)$SV40 Component III, $(^{3}H)$SV40 Component I and $(^{3}H)\lambda$ Phage DNA were obtained from Bethesda Research Laboratories, Inc. (Rockville, MD). $(\text{methyl-}^{14}\text{C})$Thymidine (51 mCi/mmole) was a product of New England Nuclear (Boston, MA). $(\text{methyl-}^{3}H)$Thymidine (43 Ci/mmole) was from International Chemical and Nuclear Corp. (Irvine, CA). $^{14}\text{C}$-nicotinic acid (56 mCi/mmole) was obtained from Amersham Corp. (Arlington Heights, IL). $(8-{^{3}H})$Adenosine-5'-triphosphate (17 Ci/mmole) was purchased from Schwarz/Mann (Orangeburg, NY). Ribonuclease A (pancreas) and snake venom phosphodiesterase were from Worthington Biochemical Corp. (Freehold, NJ). Deoxyribonuclease I (beef pancreas), nicotinamide adenine dinucleotide, nicotinamide, nicotinamide mononucleotide, adenosine 5'-diphosphoribose, adenosine-5'-triphosphate, adenosine, and nicotinic acid were from Sigma (St. Louis, MO). NAD Pyrophosphorylase
(hog liver) was a product of Boehringer Manheim (Indianapolis, IN). Adenosine-5'-monophosphate was obtained from Calbiochem (San Diego, CA). All other chemicals were reagent grade.
CHAPTER III

METHODS

Tissue Culture

Balb/c 3T3 mouse embryo fibroblasts and SV40-transformed
Balb/c 3T3 mouse embryo fibroblasts (SVT2) were grown in a
humidified 10% CO₂-air incubator at 37°C in Dulbecco's modi-

died Eagle's medium containing 10% fetal calf serum (heat-

inactivated, 30 min, 56°C), penicillin (100 units/ml), and
streptomycin (100 μg/ml). Cells to be used in experiments
were removed from liquid N₂ and subcultured twice. For
experimentation, cells were seeded at approximately 6x10³
cells/cm² in 10-cm plastic culture dishes in 13 ml of medium
containing 10% serum and antibiotics.

Nuclei Isolation

Cells were removed from dishes by treatment with 0.05%

trypsin-EDTA in phosphate buffered saline (PBS) (0.01 M

sodium phosphate buffer, pH 7.2, 0.15 M NaCl) and nuclei

were isolated according to a modification of the procedure
described by Penman (144). Approximately 1x10⁷ cells were

collected by centrifugation and washed with PBS. The cell

pellet was suspended in 2 ml of 3 mM Tris-HCl, pH 7.0,
0.5 mM MgCl₂, 3 mM NaCl. Cells were allowed to swell for 15 min and 1 ml of 0.5% (v/v) Triton X-100 solution was added to effect lysis. After 10 min, the suspension was centrifuged at 800 x g for 10 min. The crude nuclei pellet was resuspended in 2 ml of 10 mM Tris-HCl, pH 7.0, 15 mM MgCl₂, 10 mM NaCl. After 4 min, 0.3 ml detergent solution containing 6% (v/v) Tween 80 and 3% (w/v) sodium deoxycholate was added and mixed with a vortex mixer for 4 sec. After centrifugation at 800 x g for 10 min, the supernatant was removed and the nuclei pellet was washed twice with 10 mM Tris-HCl, pH 7.0, 15 mM MgCl₂, 10 mM NaCl. At this stage, the nuclei were free of microscopically-visible cytoplasmic debris. The yield of nuclei isolated by this procedure was routinely 80%.

Cell Permeabilization

Monolayer cultures of 3T3 cells were permeabilized by a slight modification of the method described by Berger and Johnson (9). The dishes of cells were chilled for 15 min, media were aspirated off, and the cells were washed twice with the permeabilization buffer containing 10 mM Tris-HCl, pH 7.8, 0.25 M sucrose, 1 mM EDTA, 1.25 mM β-mercaptoethanol and 2.5 mM MgCl₂. The cells were allowed to stand on ice for 15 min in 15 ml of ice-cold permeabilization buffer. The buffer was aspirated from the dishes, and the cells were scraped off the dish with a rubber spatula and centrifuged.
at 800 xg for 15 min. The cell pellet was resuspended in the permeabilization buffer at 2.5 x 10^7 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 percent permeabilization was determined using a hemacytometer. More than 95% of the cells were permeable, as demonstrated by the uptake of the dye.

**Poly(ADP-ribose) Polymerase Assay**

The incubation mixture (1 ml) for poly(ADP-ribose) synthesis in nuclei contained 100 mM Tris-HCl, pH 8.0, 30 mM MgCl_2, 8.2 mM NaCl, 1 mM β-mercaptoethanol, 0.2 mM EDTA, 1 mM (ade-^{14}C)NAD (1 μCi) and approximately 2 x 10^7 freshly isolated nuclei. The incubation mixture (1 ml) for poly(ADP-ribose) synthesis in permeabilized cells contained 40 mM Tris-HCl, pH 7.8, 0.8 mM EDTA, 1 mM β-mercaptoethanol, 0.2 M sucrose, 30 mM MgCl_2, 1 mM (ade-^{14}C)NAD (1 μCi) and about 2 x 10^7 permeabilized cells. Other components were added to the reaction mixture just before addition of the nuclei or cells. Incubations were carried out for 30 min at 25°C and reactions were quenched by the addition of 40% (w/v) trichloroacetic acid (TCA) (1 ml), and chilled on ice. The precipitates were washed three times with 20% (w/v) TCA and were solubilized in 1 ml 88% formic acid.
Radioactivity of the samples was determined by scintillation counting in 2 ml of aqueous scintillation cocktail (Aquasol).

Nuclease Digestion of the Poly(ADP-ribose)

The acid insoluble reaction product was washed 3 times with 20% (w/v) TCA, once with 95% ethanol, and the pellet was treated with 0.1 M NaOH for 3 hrs at 37°C to release the poly(ADP-ribose) from proteins. The insoluble material was removed by centrifugation and the pH of the supernatant was adjusted to 8.0 with 1 M HCl. The neutralized supernatant was adjusted to 50 mM in Tris and 30 mM in MgCl₂ by the addition of 0.9 M Tris·HCl, pH 7.5, and 0.6 M MgCl₂, respectively. This mixture was divided into four aliquots and aliquots were subjected to enzyme treatment with RNase (142 μg/ml) or DNase (197 μg/ml) or snake venom phosphodiesterase (500 μg/ml) at 37°C for four hours. The control incubation was not treated with enzyme. At the end of the 4-hour incubation period, a sample from each reaction mixture was removed and centrifuged at low speed and the supernatant solution was applied to a Whatman 3-mm chromatography paper. Authentic nucleotides (200 nmoles) were cochromatographed with samples. The chromatogram was first developed in 80% ethanol, air dried, and then developed in isobutyric acid: H₂O:concentrated NH₄OH (66:33:1, v/v) as previously described (53). The chromatogram was air-dried and cut into
36

1 cm strips, and the radioactivity was determined by scintillation counting in 5 ml toluene-based cocktail.

Estimation of Average Chain Length of Poly(ADP-ribose) Synthesized by Nuclei

The acid insoluble reaction product was treated with 0.25 M NaOH for 2 hrs at 37°C. The protein was precipitated by centrifugation and the supernatant was adjusted to pH 8.5 with 1 M HCl. Two-thirds of this solution was treated with an equal volume of 40% TCA, chilled and centrifuged. The TCA-insoluble pellet was washed with 95% ethanol three times and dissolved in 0.25 M NaOH. The pH of the solution was adjusted to pH 8.5 by the addition of 1 M HCl. The TCA-soluble supernatant was extracted three times with ether to remove TCA, the aqueous phase was saved, and the pH was adjusted to 8.5 with 1 M HCl. The ether-extracted supernatant, the NaOH-supernatant, and the TCA-insoluble pellet, which was dissolved in 0.25 M NaOH, were all made 50 mM in Tris and 30 mM in MgCl$_2$ by the addition of 0.9 M Tris-HCl, pH 7.5, and 0.6 M MgCl$_2$, respectively. Snake venom phosphodiesterase (200 µg/ml) was added to each aliquot and incubated at 37°C for 2 hrs. An aliquot of each reaction mixture was applied to Whatman 3-mm chromatography paper. Authentic nucleotides (200 nmoles each) were cochromatographed with samples. The chromatogram was developed first in 80% ethanol, then isobutyric acid: H$_2$O:concentrated NH$_4$OH (66:33:1, v/v) as described in the
previous section. The chromatogram was dried and cut into 1 cm fraction, and the radioactivity determined. The average chain length in the NaOH-supernatant, TCA-insoluble and TCA-soluble fractions was calculated from the following equation (151):

\[
\text{chain length} = \frac{14_{C} \text{ in PR-AMP}}{14_{C} \text{ in AMP}} + 1
\]

Preparation of \((\text{ade-}^{3}H)\text{NAD}\)

\(^3\text{H}\)-Adenine labeled NAD was prepared according to a modification of the method of Poirier et al. (146) using pig NAD pyrophosphorylase. The incubation was carried out for 60 min at 37°C in a medium containing 44.5 \(\mu\)M 3.38 mCi/\(\mu\)mole\(\text{(8-}^{3}\text{H)}\)-ATP, 4 mM NMN, 14.9 mM \(\text{MgCl}_2\), 126 mM Tris-HCl, pH 7.6, and 660 \(\mu\)g of NAD pyrophosphorylase in a final volume of 3.32 ml. The reaction was terminated by the addition of 830 \(\mu\)l of 100\% (v/v) TCA. The reaction mixture was chilled for 15 min and centrifuged at 1,000 \(\times\) g for 15 min, and the supernatant extracted with water-saturated ether five times. The residual ether was evaporated in a vacuum desiccator. The ether-free supernatant was diluted to 45 ml with 2.5 M ammonium acetate, pH 8.8 and loaded onto a 4-ml dihydroxyboryl-sepharose affinity column which had been previously equilibrated with the application buffer. The column was washed first with 45 ml of 2.5 M ammonium acetate, pH 8.8, then with 45 ml of 0.25 M ammonium acetate pH 8.8,
and finally with 10 ml of 2 mM ammonium acetate, pH 8.8. The $^{3}$H-ade$NAD$ was eluted with 12 ml of 0.1 M formic acid. Sixty five percent of the radioactivity eluted with this wash. This NAD preparation was lyophilized and dissolved in 600 µl of water. The specific activity of this NAD was 3.38 Ci/mMole. The purity was checked by chromatography on Whatman 3-mm paper with isobutyric acid:$H_{2}O$: concentrated $NH_{4}OH$ (66:33:1, v/v) as the elution solvent in the presence of ATP, ADP, NAD, AMP, adenosine and adenine as markers. Ninety six percent of the radioactivity was associated with the NAD spot.

Preparation of (carbonyl-$^{14}$C-nicotinamide)NAD

Saccharomyces cerevisiae, strain DK-8 ($^{+}$, ade$^{+}$, tyr$^{+}$, leu$^{+}$, ade$^{+}$, arg$^{+}$, ade$^{-2}$) was maintained on 1% yeast extract, 2% glucose agar plates. Cells were grown in a defined medium which contained 1.67% Bacto vitamin-free yeast extract, 1% glucose, 0.15 M adenine, and the following vitamins: 2 µg/l folic acid; 2 mg/l inositol; 2 µg/l p-aminobenzoic acid; 400 µg/l pyridoxine hydrochloride; 200 µg/l riboflavin; 400 µg/l thiamine hydrochloride and 2.24 µM nicotinic acid. Cultures were grown at 30°C on a rotary incubator. Culture growth was monitored turbidimetrically by measuring the absorbance of aliquots of culture at 600 nm in a Beckman spectrophotometer. Cultures grew with a doubling time of 100 min and reached turbidimetric densities of 4-6 at
stationary phase. Inoculums (1%) were grown for 18 hrs and were in stationary phase when experimental cultures were initiated by injecting fresh medium with a 0.2% volume of inoculum in the presence of $^{14}$C-nicotinic acid (2.24 μM, 0.15 μCi/ml culture), which was allowed to grow to a turbidimetric density of 2 A$_{600}$/ml. At this cell density, 95% of the label in the growth medium was taken up by the cells. Cells were filtered through membrane filters (0.45 μm) and washed three times with 10 ml aliquots of physiological saline. The cells were extracted with 10% (w/v) TCA by vortex mixing for about 10 sec, chilled on ice for 15 min, and centrifuged at 1,000 x g for 15 min. The supernatant was removed and extracted 5 times with H$_2$O-saturated ether and the residual ether was evaporated in a vacuum desiccator. The ether-free supernatant containing the (carbonyl-$^{14}$C)NAD was loaded onto a dihydroxyboryl-Sepharose affinity column and the NAD was purified as described previously. About 70% of the label eluted from the column with 0.1 M formic acid wash. The purity was checked by chromatography on Whatman 3-mm paper with 0.12 M sodium citrate, 2 M NH$_4$Cl, pH 4.8, 95% ethanol (25:75 v/v) as the developing solvent in the presence of NMN, Nam, Na, and NAD as markers. Ninety-seven percent of the radioactivity was associated with the NAD spot.
Purification of Snake Venom Phosphodiesterase on Blue Sepharose

Commercial snake venom phosphodiesterase was purified by a slight modification of the method described by Oka et al. (138). Briefly, snake venom phosphodiesterase purchased from Worthington (5 mg) was dissolved in 1.5 ml of 5 mM potassium phosphate containing 10 mM Tris-HCl and 50 mM NaCl, pH 7.5, and applied to a 0.7 x 8 cm Blue Sepharose column. The column was washed with 20 ml of application buffer and then with 20 ml of 10 mM potassium phosphate containing 10 mM Tris-HCl, 50 mM NaCl, pH 7.5 and finally with 20 ml of 30 mM potassium phosphate containing 10 mM Tris-HCl, 50 mM NaCl, pH 7.5 to remove the nonspecific phosphatase and 5'-nucleotidase activities. Phosphodiesterase was then eluted by elevating the phosphate concentration to 100 mM. The recovery of phosphodiesterase with this wash was 70% and the contamination of phosphatases was reduced to less than 1/160 of the original. Fractions were pooled, made 20% in glycerol, and concentrated to 0.7 ml with a millipore concentrator (10,000 MW cut-off). The concentrated enzyme preparation was dialyzed against 30 mM potassium phosphate buffer, pH 7.5, containing 10 mM Tris-HCl, 50 mM NaCl and 20% glycerol and stored frozen.

The assay mixture for phosphodiesterase contained 99 mM Tris-HCl, pH 8.9, 99 mM NaCl, 13.5 mM MgCl₂, 0.5 mM p-nitrophenyl thymidine-5'-phosphate and enzyme in 1 ml. The reaction was started by the addition of enzyme and the
increase in absorbance at 400 nm was monitored at 25°C. One unit was defined as the activity that hydrolyzes one μmole of p-nitrophenyl thymidine-5'-phosphate per min to liberate p-nitrophenol ($\varepsilon_{400} = 16,000 \text{ cm}^2 \text{ mole}^{-1}$) under the conditions employed.

Estimation of Average Chain Length of the Poly(ADP-ribose) Synthesized by Permeable 3T3 Cells

Logarithmically growing 3T3 cells at a density of 4 x 10^4 cells/cm^2 were permeabilized as previously described. Ten million permeable cells were incubated in a total volume of 180 μl of complete poly(ADP-ribose) polymerase reaction mixture containing 1 mM (ade^3-H)NAD (11 μCi). After 5, 15, and 30 min of incubation at 25°C, the reaction was terminated by the addition of 20 μl of 100% TCA. The acid insoluble pellet was washed three times with 20% TCA and once with ether. The cell pellets were suspended in 10 ml of 0.1 M potassium phosphate buffer, pH 8.6 containing 6 M guanidine, sonicated for 15 min, and centrifuged at 21,000 x g for 20 min to remove debris. The supernatant was adjusted to pH 8.6 with 8 M KOH and loaded on a 0.8-ml dihydroxyboryl-Sepharose affinity column (165) preequilibrated with the application buffer. After application of the samples, columns were washed consecutively with 10 ml of guanidine buffer, 10 ml of 10 mM sodium morpholine propane sulfonic acid (MOPS) buffer, pH 8.5, 30 ml of 20 mM
sodium citrate buffer, pH 4.5 and finally with 10 ml of MOPS buffer. The columns were then incubated for 3 hrs at 37°C with 200 µl of 20 mM potassium phosphate buffer pH 7.5 containing 1 mM MgCl₂ and 0.05 units of purified snake venom phosphodiesterase. Nucleotides were eluted from the column with 3 ml of water. The enzyme was precipitated with TCA, using bovine serum albumin as a co-precipitant, chilled on ice, and centrifuged. The supernatant was extracted with H₂O-saturated ether 5 times and the residual ether was evaporated in a vacuum desiccator. The ether-free supernatant was lyophilized, taken up in a total volume of 300 µl of H₂O, and applied to Whatman 3 mm chromatography paper. The chromatogram was developed with isobutyric acid:H₂O: concentrated NH₄OH (66:33:1, v/v) in the presence of ATP, AMP, PR-AMP and adenosine as markers. The average chain length was estimated as described in the previous section.

Treatment of Cells With MNNG In Vivo

Cultures of 3T3 cells in the log phase of growth were incubated for 30 min at 37°C in medium containing 50 µg/ml MNNG. MNNG was dissolved in media lacking serum just before use.

NAD Degradation in Permeable Cells

Poly(ADP-ribose) polymerase, NAD glycohydrolase and NAD pyrophosphatase activities were assayed simultaneously
in nucleotide permeable cells. The incubation mixture for enzyme assays (0.075 ml) contained 40 mM Tris-HCl, pH 7.8, 0.8 mM EDTA, 1 mM β-mercaptoethanol, 0.2 M sucrose, 30 mM MgCl₂, 1 mM NAD⁺ containing 0.8 μCi(ade⁻³H)NAD⁺ and 0.06 μCi (nicotinamide⁻¹⁴C)NAD and approximately 3 x 10⁶ permeable cells. Incubations were at 25°C for 10, 20, and 30 min. Reactions were terminated by adjusting to 20% in TCA and chilling on ice. The precipitates were washed 3 times with 20% TCA and solubilized with 0.1 ml of 88% formic acid. Radioactivity of the samples was determined by scintillation counting in 2 ml Aquasol. The supernatant from each incubation was extracted 5 times with an equal volume of diethyl ether and subjected to paper chromatography in the system of Witholt (200) to separate nicotinamide and NMN from other radiolabelled compounds. Radioactivity associated with nicotinamide and NMN was determined by scintillation counting in a toluene based cocktail.

Determination of the Rate of DNA Synthesis

To determine the rate of DNA synthesis, 35-mm dishes of cells were exposed to 1 μCi/ml of (methyl⁻³H)thymidine (50 Ci/m mole) for 30 min at 37°C. The medium was aspirated and the cells were washed five times with PBS. The cells were dissolved with 0.5 ml of 0.1 M NaOH and 1 mM nicotinamide and combined with an additional 0.5 ml wash of the dish. The combined extracts were mixed and chilled, and 0.1 ml
of 100% (w/v) TCA was added. After standing on ice for 15 min, the acid insoluble material was collected by centrifugation. The pellet was washed three times with ice-cold 20% (w/v) TCA and dissolved in 0.5 ml of 88% formic acid. A portion was placed in an aqueous scintillation cocktail (Aquasol) for counting.

Alkaline Sucrose Density Gradients

Logarithmically growing 3T3 cells were radiolabelled by the addition of 0.15 μCi/ml of (methyl-\(^{14}\)C)-thymidine or 0.4 μCi/ml of (methyl-\(^{3}\)H)-thymidine with incubation for 24 hours. Cells labelled with \(^{3}\)H-thymidine were used as internal controls and those labelled with \(^{14}\)C-thymidine were treated with compound. Cells were removed from the dish with 0.25 ml of Puck's saline containing 0.02% (w/v) EDTA (148) and 0.05 ml each of \(^{14}\)C-thymidine and \(^{3}\)H-thymidine labelled cell suspensions were layered onto each gradient. A total of approximately \(2 \times 10^5\) cells was applied to each gradient. For experiments involving treatment with DNase I \textit{in vitro}, the prelabelled cells were permeabilized and incubated with the standard poly(ADP-ribose) polymerase assay reaction mixture containing 1 mM unlabelled NAD and different concentrations of DNase I, at 25°C. After 10 min incubation, the reaction mixture was chilled, adjusted to 30 mM in EDTA and centrifuged. The cell pellet was suspended in Puck's saline containing
0.02% (w/v) EDTA, and about $1 \times 10^5$ cells were layered on each gradient. Gradients were 5-20% (w/v) sucrose, 0.5 M NaOH, 0.05 M EDTA, 0.5 M NaCl; 4.3 ml total volume. On the bottom of each gradient was a 0.4 ml 40% (w/v) alkaline sucrose shelf. First, 100 µl of lysis buffer (0.5 M NaOH, 0.05 M EDTA, 0.5 M NaCl, 1% (v/v) Triton X-100) was layered onto each gradient immediately before layering the cell suspensions. Following the cell suspension, 100 µl of lysis buffer was layered. The gradients were kept in the dark for 12 hrs at room temperature, followed by centrifugation in a Beckman SW 50.1 rotor at 14,000 rpm for 12 hrs. Fractions were collected from the top of the gradients onto 2.4 cm Whatman 3-mm filter paper disks. The filter paper disks were washed once in 20% TCA, twice in 10% TCA and twice in 95% ethanol. Each wash was for 10 min on ice. The filter paper disks were dried and counted using a toluene-based liquid scintillation cocktail. Recovery of radioactivity was routinely greater than 80%.

DNA Molecular Weight Determinations

SV40 Component I (53S), SV40 Component III (16 S) and λ Phage DNA (40.5 S) were used for the determination of the constant $\beta$ of the formula:

$$S_{20,w} = \frac{\beta D}{(\text{rev/min})^2 \times t}$$

(112,113)
where $D$ is the sedimentation distance in cm, $t$ is time in hr, and $S$ is the sedimentation coefficient. Then, $S$ values corresponding to each fraction of the gradient were calculated and used to determine the DNA molecular weight of the fractions according to the expression:

$$S_{20,w}^o = 0.0528 \times M_r^{0.4}$$  \hspace{1cm} (180)

where $S_{20,w}^o$ is the sedimentation coefficient expected in a solution having the viscosity and density of water at 20°C.

Weight average molecular weight ($M_w$) were calculated for the regions of the gradient enclosing the peaks. They were obtained by using the expression:

$$M_w = \frac{\sum C_i M_r}{\sum C_i}$$

where $C_i$ is cpm of the fraction and $M_r$ is the fractional molecular weight. Number of single-strand breaks were calculated with the formula:

$$\frac{M_{w,i}}{M_{w,b}} - 1$$

where $M_{w,i}$ is weight-average molecular weight of control DNA and $M_{w,b}$ of the broken DNA (50).
Spectrophotometric Determinations of RNA, DNA and Protein

Cells were diluted in phosphate-buffered saline, adjusted to 5% (w/v) in HClO₄ by the addition of 10% (w/v) HClO₄ and chilled on ice for 15 min. The solution was centrifuged in the cold at 1500 x g for 10 min. The pellet was dissolved in 0.25 M NaOH. This solution was used for RNA, DNA, and protein determinations.

RNA was hydrolyzed (0.25 M NaOH, 37°C, 1 hr) and the hydrolyzate was chilled on ice for 15 min. The hydrolyzate was adjusted to 5% (w/v) in HClO₄ by the addition of cold 12% (w/v) HClO₄ and the samples were centrifuged at 1500 x g for 10 min. RNA concentrations were obtained by measurements at 260 nm of the material which becomes soluble in cold 5% (w/v) HClO₄ following alkaline hydrolysis using 34 as the absorbance obtained for 1 mg/ml RNA (108).

DNA assays were done on the material which was insoluble in cold 5% (w/v) HClO₄ following alkaline hydrolysis. The pellets were suspended in 5% (w/v) HClO₄ and incubated at 70°C for 30 min to hydrolyze DNA. Samples were chilled on ice for 15 min, and centrifuged at 1500 x g for 10 min. DNA concentrations were obtained by measurements at 260 nm of the supernatant, using 30 as the absorbance obtained for 1 mg/ml of DNA (108).

Protein was determined by the binding of Coomassie Brilliant Blue G-250 to protein (20) using 1 mg/ml BSA in 0.25 M NaOH as standard.
DNA Determination by Diphenylamine Assay

Cells were diluted in PBS and adjusted to 0.5 M HClO₄ by the addition of 1 M HClO₄. The solution was chilled on ice for 15 min, and centrifuged at 1500 x g for 10 min. The pellet was washed once with 0.5 M HClO₄ and then incubated in 0.5 M HClO₄ at 70°C for 30 min to hydrolyze DNA. The hydrolyzate was chilled on ice for 15 min and centrifuged at 1500 x g for 10 min. The supernatant was adjusted to 1.5 M in HClO₄ by the addition of 3 M HClO₄. DNA was determined by Richards' method (152), except that the reagent contained 3% (w/v) diphenylamine and 0.01% paraldehyde in glacial acetic acid and the test solution was combined with the reagent at 1:1 ratio.

Protein Determination

Protein concentrations were determined by the Lowry et al. (109) procedure, using BSA as standard.
CHAPTER IV

RESULTS

Isolation and Characterization of Nuclei
from 3T3 and SVT2 Cells

Most poly(ADP-ribose) polymerase assays have been
conducted with isolated cell nuclei or chromatin, since
the substrate, NAD, cannot enter intact cells. I have
isolated nuclei from normal and transformed 3T3 cells
utilizing a slightly modified double-detergent method
described by Penman (144). Based on DNA content, the yield
of nuclei isolated by this technique was routinely 80%.
The protein and RNA content of the intact cells and isolated
cell nuclei is shown in Table I. Logarithmically growing
3T3 cells had a higher protein and RNA content per cell than
the stationary 3T3 cells. At similar cell densities, 3T3
cells had higher levels of protein and RNA per cell than
logarithmically growing SVT2 cells. Approximately 75% of
the protein and RNA was removed from both 3T3 and SVT2 cells
upon nuclei isolation. When visualized under the light
microscope, the nuclei were free of cytoplasmic debris. Table
I also shows that the DNA content in 3T3 cells and in their
transformed counterparts is very similar.
TABLE I

DNA, PROTEIN AND RNA CONTENT OF 3T3 CELLS AND ISOLATED CELL NUCLEI

<table>
<thead>
<tr>
<th>CELL LINE</th>
<th>GROWTH PHASE</th>
<th>DNA</th>
<th>PROTEIN</th>
<th>RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>3T3 Cells</td>
<td>LOG</td>
<td>21.8±0.9</td>
<td>470±62.0</td>
<td>38.3±0.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(n=2)</td>
<td>(n=4)</td>
<td>(n=2)</td>
</tr>
<tr>
<td>Nuclei</td>
<td>LOG</td>
<td>132±42.9</td>
<td></td>
<td>12.4±2.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(n=6)</td>
<td></td>
<td>(n=3)</td>
</tr>
<tr>
<td>Cells</td>
<td>STATIONARY</td>
<td>21.7±1.9</td>
<td>399±47.9</td>
<td>21.1±0.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(n=2)</td>
<td>(n=4)</td>
<td>(n=2)</td>
</tr>
<tr>
<td>Nuclei</td>
<td>STATIONARY</td>
<td>111±23.4</td>
<td></td>
<td>11.2±2.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(n=5)</td>
<td></td>
<td>(n=3)</td>
</tr>
<tr>
<td>SVT2 Cells</td>
<td>LOG</td>
<td>17.2±1.2</td>
<td>305±36.4</td>
<td>34.4±5.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(n=5)</td>
<td>(n=2)</td>
<td>(n=2)</td>
</tr>
<tr>
<td>Nuclei</td>
<td>LOG</td>
<td>71.9±15.6</td>
<td></td>
<td>8.0±1.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(n=4)</td>
<td></td>
<td>(n=4)</td>
</tr>
</tbody>
</table>

Nuclei isolation from 3T3 and SVT2 cells was carried out as described in METHODS. RNA, DNA, and protein content in cells and isolated cell nuclei was determined spectrophotometrically. Standard deviations are shown. The number of determinations is shown in parentheses.
Characterization of Poly(ADP-ribose) Synthesis in Isolated Cell Nuclei

The main purpose of this work was to examine poly(ADP-ribose) synthesis in 3T3 and SVT2 cells under various physiologic conditions. Therefore, I first characterized poly(ADP-ribose) synthesis in isolated cell nuclei. Upon incubation with (ade-14C) NAD, isolated cell nuclei synthesized an acid-insoluble material. To confirm that the material was poly(ADP-ribose), the acid-insoluble product was examined for its susceptibility to snake venom phosphodiesterase (SVPD) (Fig. 3). More than 90% of the labelled material was degraded by SVPD yielding AMP and 2'-5'-phosphoribosyl-5'-AMP (PR-AMP) as the degradation products as demonstrated by paper chromatography. However, the acid-insoluble product was not degraded by DNase I or RNase (Table II). The resistance to DNase I and RNase, and the susceptibility to SVPD indicate that a great majority of the material synthesized by these nuclei was poly(ADP-ribose). We cannot exclude the possibility that a small amount of mono(ADP-ribose) was also synthesized by these nuclei. To demonstrate that the material was synthesized by poly(ADP-ribose) polymerase, known inhibitors of the enzyme were tested (Table III). At concentrations equimolar to the substrate, NAD (1 mM), thymidine, nicotinamide, theophylline and caffeine inhibited synthesis by 55%, 59%, 74% and 33%, respectively. Nicotine, nicotinate and
Identification of snake venom phosphodiesterase digestion products of material synthesized by 3T3 cell nuclei. The acid-insoluble product synthesized by isolated 3T3 cell nuclei was treated with snake venom phosphodiesterase and the digestion products were identified by paper chromatography as described in METHODS. The figure shows the percentage of the total counts associated with each strip on the chromatogram. Control incubation, (o-o); incubation in the presence of snake venom phosphodiesterase, (●-●).
<table>
<thead>
<tr>
<th></th>
<th>Treatment with SVPD</th>
<th>Treatment with DNase</th>
<th>Treatment with RNase</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ORIGIN</td>
<td>94.5</td>
<td>8.6</td>
<td>91.8</td>
</tr>
<tr>
<td>PR-AMP</td>
<td>0.3</td>
<td>77.1</td>
<td>2.7</td>
</tr>
<tr>
<td>AMP</td>
<td>5.2</td>
<td>14.3</td>
<td>5.5</td>
</tr>
</tbody>
</table>

Nuclei from 3T3 cells (4.5 x 10^7 cells at a density of 9 x 10^4 cells/cm^2) were isolated and incubated in a total volume of 0.5 ml and 1 mM (ade-^{14}C)NAD at 25°C for 30 min. The acid-insoluble reaction product was treated with nucleases as described in METHODS. The table shows the percentage of the total radioactivity associated with the origin, PR-AMP and AMP spots after each treatment.
TABLE III

EFFECT OF PYRIDINE AND PURINE DERIVATIVES ON POLY(ADP-RIbose) POLYMERASE ACTIVITY IN 3T3 CELL NUCLEI

<table>
<thead>
<tr>
<th>Additions</th>
<th>Poly(ADP-ribose) polymerase activity (% control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>Thymidine</td>
<td>45</td>
</tr>
<tr>
<td>Nicotinamide</td>
<td>41</td>
</tr>
<tr>
<td>Nicotine</td>
<td>104</td>
</tr>
<tr>
<td>Nicotinate</td>
<td>102</td>
</tr>
<tr>
<td>Quinolinate</td>
<td>93</td>
</tr>
<tr>
<td>Theophylline</td>
<td>26</td>
</tr>
<tr>
<td>Caffeine</td>
<td>67</td>
</tr>
<tr>
<td>3-Isobutyl-1-methylxanthine</td>
<td>87</td>
</tr>
<tr>
<td>Kinetin(6-furfurylaminopurine)</td>
<td>87</td>
</tr>
<tr>
<td>Kinetin riboside (6-furfurylaminopurine riboside)</td>
<td>70</td>
</tr>
<tr>
<td>N6-(Δ2-isopentenyl)-adenine</td>
<td>55</td>
</tr>
<tr>
<td>N6-(Δ2-isopentenyl)-adenosine</td>
<td>75</td>
</tr>
</tbody>
</table>

Log phase 3T3 cell nuclei containing 17.6 μg DNA were incubated at 25°C with 1 mM (ade-3H)NAD (0.8 μCi) in a total volume of 0.1 ml. The poly(ADP-ribose) polymerase activity is shown for a 10-min incubation. Inhibitors were added to the reaction components just before addition of the nuclei and all inhibitors were present at 1 mM. The amount of poly(ADP-ribose) synthesized in the presence of the inhibitors is expressed as a percentage of the amount synthesized in the control reaction. DNA was determined by diphenylamine assay. Control incubation contained 25 pm moles ADP-ribose converted to poly(ADP-ribose)/min/10⁶ nuclei. Duplicate analyses agreed within 5%.
quinolinate were not inhibitory. Since theophylline and caffeine are also inhibitors of cAMP phosphodiesterase, the effect of other known inhibitors of this enzyme were also tested on the activity of poly(ADP-ribose) polymerase. As shown in Table III, isopentenyladenine inhibited the enzyme by 45%, kinetin riboside by 30% and isopentenyladenosine by 25%. Kinetin was only slightly inhibitory.

**Optimization of Poly(ADP-ribose) Reaction Conditions**

As shown in Figure 4 in the presence of 1 mM NAD the synthesis of poly(ADP-ribose) by nuclei was linear for at least 20 min and reached a maximum level at 50 min. In subsequent experiments, incubations were carried out only for 10 min to insure that the amount of incorporation was linear with respect to time. Figure 5 shows that the synthesis of the polymer was proportional to the number of nuclei in the reaction mixture between $1 \times 10^7$ and $10 \times 10^7$ nuclei/ml. In all the subsequent experiments, the number of nuclei utilized fell in this range. Figure 6 shows the effect of MgCl$_2$ on the activity of the poly(ADP-ribose) polymerase. The optimum MgCl$_2$ concentration fell in the range of 10 to 30 mM. In experiments to be described later, 30 mM MgCl$_2$ was utilized. Several reports have shown that the synthesis of poly(ADP-ribose) is stimulated by agents which are known to cause fragmentation of DNA. Stimulation of poly(ADP-ribose) synthesis by DNase I has previously been reported.
Synthesis of poly(ADP-ribose) in isolated 3T3 cell nuclei as a function of time. The reaction mixture (0.1 ml) contained 100 mM Tris-HCl (pH 8.0), 8.2 mM NaCl, 1 mM β-mercaptoethanol, 0.2 mM EDTA, 30 mM MgCl₂, 1 mM (ade⁻³H)NAD (0.8 μCi) and 2 x 10⁶ nuclei, isolated from 3T3 cells at a density of 8 x 10⁴ cells/cm². Poly(ADP-ribose) synthesis is expressed as pmoles of (ade⁻³H)ADP-ribose incorporated per 10⁶ nuclei. DNA content was determined by diphenylamine assay.
Figure 5

Relation of poly(ADP-ribose) synthesis to number of isolated cell nuclei in reaction. The reaction mixture contained 100 mM Tris-HCl (pH 8.0), 2 mM MgCl$_2$, 8.2 mM NaCl, 1 mM β-mercaptoethanol, 0.2 mM EDTA, 1 mM (ade$^{14}$C)NAD (0.1 μCi) and the indicated number of isolated SVT2 nuclei in a final volume of 0.1 ml. Reactions were incubated at 25°C for 10 min.
Effect of MgCl$_2$ concentration of poly(ADP-ribose) synthesis in isolated 3T3 cell nuclei. The reaction mixture contained 100 mM Tris-HCl (pH 8.0), 8.2 mM NaCl, 1 mM β-mercapotethanol, 0.2 mM EDTA, 1 mM (ade-$^{14}$C)NAD (0.1 μCi), 3 x 10$^6$ nuclei and the indicated amount of MgCl$_2$ in a final volume of 0.1 ml. Reactions were incubated at 25°C for 10 min.
INTEGRATION OF APP-RIBOSE
by Miller (116) in HeLa cell nuclei. I examined the response of poly(ADP-ribose) polymerase in 3T3 cell nuclei to DNase I (Fig. 7). Treatment of nuclei with DNase I caused up to a 5-fold increase in poly(ADP-ribose) synthesis. As the amount of DNase I in the incubation mixture was increased, the rate of synthesis of the polymer increased until it reached a maximum level. The maximal stimulation in the synthesis of the polymer by DNase I occurred with a concentration of 0.5 mg/ml.

The stability of poly(ADP-ribose) synthesized by isolated nuclei was examined (Table IV). Isolated nuclei were incubated with (ade-14C) NAD for 30 min at 25°C as described in METHODS. At the end of the incubation period, the nuclei were collected by centrifugation at 4°C and resuspended in the reaction mixture for poly(ADP-ribose) synthesis except that the NAD was unlabelled. The resuspended nuclei were incubated for an additional 30 min. The reaction mixture was precipitated with 20% (w/v) TCA and processed as described in METHODS. Table IV demonstrates that the polymer synthesized by isolated nuclei is quite unstable; 53% of the product is degraded after 30 min incubation with 1 mM unlabelled NAD.

The effect of various concentrations of NAD on the catalytic activity of poly(ADP-ribose) polymerase was studied. Figure 8 shows Lineweaver-Burk plots for the
Synthesis of poly(ADP-ribose) in isolated SVT2 cell nuclei in the presence of DNase I. The reaction mixture (0.1 ml) contained $3 \times 10^6$ nuclei in 100 mM Tris-HCl (pH 8.0), 8.2 mM NaCl, 1 mM β-mercaptoethanol, 0.2 mM EDTA, 30 mM MgCl$_2$, 1 mM (ade-$^{14}$C)NAD (0.1 μCi) and the indicated amount of DNase I. Reactions were incubated at 25°C for 10 min.
INTEGRATION OF ADP-RIBOSE
### TABLE IV

**STABILITY OF POLY(ADP-RIbose) IN ISOLATED 3T3 CELL NUCLEI**

<table>
<thead>
<tr>
<th>Poly(ADP-ribose) (pmoles of ADP-ribose/10^6 nuclei)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 min incubation with (ade-14C)NAD</td>
</tr>
<tr>
<td>886</td>
</tr>
<tr>
<td>Additional 30 min incubation with unlabelled NAD</td>
</tr>
<tr>
<td>418</td>
</tr>
</tbody>
</table>

Nuclei (1 x 10^6) were isolated from 3T3 cells at a density of 8 x 10^4 cells/cm^2 and incubated in the complete poly(ADP-ribose) polymerase reaction mixture (0.1 ml) containing 1 mM (ade-14C)NAD (0.1 μCi) at 25°C.
Figure 8

Determination of the apparent Michaelis-Menten constant. Poly(ADP-ribose) polymerase in isolated 3T3 and SVT2 cell nuclei was assayed as described in METHODS in the presence of various concentrations of NAD. Initial velocities were determined from 4 min incubations at 25°C. The $K_m$ values for NAD were calculated by a computer program as described (193). Panel A: 3T3 cell nuclei; Panel B: SVT2 cell nuclei.
poly(ADP-ribose) polymerase is isolated cell nuclei. The apparent Michaelis-Menten constants ($K_m$) for NAD$^+$ obtained for enzyme activities in 3T3 and SVT2 cell nuclei were 75 μM and 124 μM, respectively.

**Poly(ADP-ribose) Polymerase Activity in Isolated Nuclei as a Function of Growth**

In the growth experiments to be described below, subconfluent 3T3 and SVT2 cells were subcultured at about $6 \times 10^4$ cells/dish in 35-mm plastic culture dishes in 2 ml fresh Dulbecco's modified Eagle's medium containing 10% fetal calf serum and allowed to grow in a humidified 10% CO$_2$-air incubator at 37°C. Growth medium was replaced every other day throughout the experiments. As shown in Figure 9, 3T3 cell numbers increased logarithmically for five generations with a doubling time of approximately 26 hours until they reached a density of about $1 \times 10^6$ cells/dish. At this density, the rate of increase in cell number slowed down and then stopped at about $1.5 \times 10^6$ cell/dish as the cells exhibited density-dependent inhibition of growth (DDIG) despite replenishment with fresh medium. SVT2 cells, however, grew logarithmically with a generation time of approximately 17 hours until they reached a density 5 times higher than that at which 3T3 cells showed DDIG.

The activity of poly(ADP-ribose) polymerase in normal and transformed 3T3 cell nuclei was examined as a function
Figure 9

Growth of 3T3 and SVT2 cells. 3T3 cells at near confluency and log phase SVT2 cells were seeded in 35-mm petri dishes at about $6 \times 10^4$ cells/dish in 2 ml medium containing 10% fetal calf serum and cultivated at 37°C. Media were replaced with fresh media every 48 hours. At the times indicated, the cells were harvested and cell numbers were determined with an electronic particle counter. 3T3 cells, (●-●); SVT2 cells, (○-○).
of growth (Table V). The enzyme activity in isolated 3T3 cell nuclei exhibited a gradual decrease as cells entered higher densities. When measured in SVT2 cells, however, no significant change in enzyme activity was observed as a function of cell density, and the enzyme activity in transformed cell nuclei was similar to that of stationary phase 3T3 cell nuclei.

Since poly(ADP-ribose) polymerase activity in nuclei isolated from stationary phase 3T3 cell cultures had 50% of the activity observed in nuclei isolated from logarithmically growing 3T3 cells, it was of interest to see if shorter chains of poly(ADP-ribose) could account for this reduced activity. To answer this question, the average chain length of the polymer synthesized by isolated 3T3 and SVT2 cell nuclei was determined as a function of growth (Table VI). The TCA-insoluble reaction product was released from protein by treatment with 0.25 M NaOH for 2 hrs at 37°C. The protein was precipitated by centrifugation and 1/3 of the supernatant was utilized in the determination of the total average chain length of the product. The remaining 2/3 was treated with 40% (w/v) TCA and centrifuged. The TCA-insoluble fraction was utilized for the average chain length determination of the long-chain polymer, whereas the TCA-soluble fraction was employed to determine the average chain length of the
### TABLE V

POLY(ADP-RIbose) POLYMERASE ACTIVITY IN 3T3 and SVT2 CELL NUCLEI AS A FUNCTION OF GROWTH

<table>
<thead>
<tr>
<th>CELL DENSITY (cells/cm²)</th>
<th>GROWTH STAGE</th>
<th>POLY(ADP-RIbose) POLYMERASE ACTIVITY (pmoles of ADP-ribose/min/10⁶ nuclei)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3T3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.5 x 10⁴</td>
<td>Mid Log</td>
<td>57.8</td>
</tr>
<tr>
<td>9.3 x 10⁴</td>
<td>Late Log</td>
<td>40.4</td>
</tr>
<tr>
<td>2.3 x 10⁵</td>
<td>Stationary</td>
<td>24.8</td>
</tr>
<tr>
<td>SVT2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.4 x 10⁴</td>
<td>Log</td>
<td>25.8</td>
</tr>
<tr>
<td>1.9 x 10⁵</td>
<td>Log</td>
<td>24.4</td>
</tr>
<tr>
<td>5.8 x 10⁵</td>
<td>Log</td>
<td>29.6</td>
</tr>
</tbody>
</table>

Nuclei were isolated from 3T3 and SVT2 cells at indicated cell densities and incubated with 1 mM (ade⁻¹⁴)NAD under optimum conditions for 10 min. The enzyme activity is expressed as pmoles of (ade⁻¹⁴C)ADP-ribose converted into acid-insoluble product per min per 10⁶ nuclei. The values shown are the average of duplicate analyses. Duplicate analyses agreed within 10%.
<table>
<thead>
<tr>
<th>CELL DENSITY (cells/cm^2)</th>
<th>GROWTH STAGE</th>
<th>TOTAL</th>
<th>TCA-insoluble</th>
<th>TCA-soluble</th>
<th>Average Chain Length (( \frac{PR-AMP}{AMP} + 1 ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>3T3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7 x 10^4</td>
<td>Log</td>
<td>3.8</td>
<td>9.8 (61%)</td>
<td>3 (39%)</td>
<td></td>
</tr>
<tr>
<td>2 x 10^5</td>
<td>Stationary</td>
<td>2.9</td>
<td>13 (36%)</td>
<td>2.4 (64%)</td>
<td></td>
</tr>
<tr>
<td>SVT2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.2 x 10^4</td>
<td>Log</td>
<td>6.0</td>
<td>16.3 (41%)</td>
<td>4.3 (59%)</td>
<td></td>
</tr>
<tr>
<td>2.8 x 10^5</td>
<td>Log</td>
<td>5.0</td>
<td>14.3 (54%)</td>
<td>3.5 (46%)</td>
<td></td>
</tr>
</tbody>
</table>

The chain length of the poly(ADP-ribose) synthesized by isolated 3T3 and SVT2 cell nuclei was determined as described in METHODS. The numbers in parentheses show the percentage of the total counts in TCA-soluble and TCA-insoluble fractions. Duplicate analyses agreed within 7%.
short-chain oligomers. In logarithmically growing cell cultures, the total average chain length of the polymer synthesized by isolated cell nuclei was estimated to be 3.8 residues long, which was 50% longer than the stationary phase cell nuclei. Furthermore, the product synthesized by the nuclei isolated from logarithmically growing cells consisted mostly of the long-chain poly(ADP-ribose), whereas the polymer synthesized by stationary phase cell nuclei had mostly short chains. In transformed cell nuclei, the total average chain length was longer than that of the normal cell nuclei but very similar in low and high density SVT2 cells. The percentage of the total label was equally distributed between the TCA-insoluble and TCA-soluble fractions with average chain lengths of 14.3 to 16.3, and 3.5 to 4.3, respectively.

Poly(ADP-ribose) Synthesis in Permeable Cells

In this part of the study, I have utilized a permeable cell system for the examination of poly(ADP-ribose) synthesis in normal and transformed monolayer 3T3 cell cultures. The permeable cell technique was chosen with the expectation that this system might maintain more of the characteristics of the intact cell. Indeed, on light-microscopic examination, the permeabilized cells appeared swollen but morphologically intact. Upon incubation with (ade$^{14}$C)NAD, the permeabilized 3T3 cells synthesized a TCA-insoluble product.
As shown in Table VII, the polymer was resistant to the action of both DNase I and RNase, but was converted to AMP and PR-AMP by treatment with SVPD, confirming the identity of the polymer as poly(ADP-ribose). Further characterization of poly(ADP-ribose) polymerase was achieved by examining its susceptibility to known inhibitors (Table VIII). At equimolar concentrations to NAD (1 mM), thymidine, nicotinamide and 3-aminobenzamide inhibited poly(ADP-ribose) synthesis by 49%, 47%, and 82%, respectively. Nicotine, nicotinic acid, quinolinic acid, caffeine and isobutyl-methylxanthine did not inhibit the enzyme.

Contrary to the results obtained with isolated nuclei, none of the four cytokinins tested inhibited poly(ADP-ribose) polymerase in the permeable cell system.

Optimization of Poly(ADP-ribose) Reaction Conditions

The assay conditions for poly(ADP-ribose) polymerase reaction in permeable 3T3 cells were optimized. As shown in Figure 10, the synthesis of the polymer was proportional to the number of cells in the reaction mixture between $1 \times 10^7$ and $10 \times 10^7$ cells/ml. Approximately $2 \times 10^7$ cells/ml were utilized in subsequent experiments. The effect of MgCl$_2$ concentration on poly(ADP-ribose) synthesis was examined (Figure 11). Synthesis of the polymer was stimulated several-fold by MgCl$_2$ up to 30 mM. Above 30 mM concentrations of MgCl$_2$, the synthesis of the polymer exhibited a sharp decrease. The effect of MgCl$_2$ on polymer synthesis
TABLE VII

NUCLEASE TREATMENT OF POLY(ADP-RIbose) POLYMERASE REACTION PRODUCT FROM PERMEABLE 3T3 CELLS

<table>
<thead>
<tr>
<th></th>
<th>CONTROL</th>
<th>TREATMENT WITH</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>SVPD</td>
<td>DNase</td>
<td>RNase</td>
</tr>
<tr>
<td>(Percent of Total cpm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ORIGIN</td>
<td>91.3</td>
<td>9.5</td>
<td>87.6</td>
<td>81.5</td>
</tr>
<tr>
<td>PRE-AMP</td>
<td>1.2</td>
<td>75</td>
<td>4.3</td>
<td>5.4</td>
</tr>
<tr>
<td>AMP</td>
<td>7.5</td>
<td>15.5</td>
<td>7.6</td>
<td>13</td>
</tr>
</tbody>
</table>

Log phase 3T3 cells (3 x 10^7 cells) at a density of 9 x 10^4 cells/cm^2 were permeabilized and incubated in a total volume of 1 ml with 1 mM (ade-14C)NAD (2 μCi) in the presence of DNase I (1 mg/ml) at 25°C for 30 min. The acid-insoluble reaction product was treated with nuclease as described in METHODS.
TABLE VIII  
EFFECT OF PYRIDINE AND PURINE DERIVATIVES ON POLY(ADP-RIBOSE) POLYMERASE ACTIVITY IN PERMEABLE 3T3 CELLS

<table>
<thead>
<tr>
<th>Additions</th>
<th>Poly(ADP-ribose) Polymerase Activity (% Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>Thymidine</td>
<td>51</td>
</tr>
<tr>
<td>Nicotinamide</td>
<td>40</td>
</tr>
<tr>
<td>Nicotine</td>
<td>93</td>
</tr>
<tr>
<td>Nicotinate</td>
<td>97</td>
</tr>
<tr>
<td>Quinolinate</td>
<td>92</td>
</tr>
<tr>
<td>Theophylline</td>
<td>59</td>
</tr>
<tr>
<td>Theobromine</td>
<td>53</td>
</tr>
<tr>
<td>Caffeine</td>
<td>89</td>
</tr>
<tr>
<td>3-Isobutyl-1-methylxanthine</td>
<td>91</td>
</tr>
<tr>
<td>Kinetin (6-furfurylamino purine)</td>
<td>95</td>
</tr>
<tr>
<td>Kinetin riboside (6-furfurylamino purine riboside)</td>
<td>96</td>
</tr>
<tr>
<td>N6-(Δ2-isopentenyl)-adenine</td>
<td>92</td>
</tr>
<tr>
<td>N6-(Δ2-isopentenyl)-adenosine</td>
<td>107</td>
</tr>
<tr>
<td>3-aminobenzamide</td>
<td>18</td>
</tr>
<tr>
<td>3-amino benzoic acid</td>
<td>100</td>
</tr>
<tr>
<td>Ap₄ A(diadenosine tetraphosphate)</td>
<td>93</td>
</tr>
</tbody>
</table>

2 x 10⁶ log phase 3T3 cells (1.2 x 10⁵ cells/cm²) were permeabilized and incubated at 25°C with 1 mM (ade⁻³H)NAD (0.8 μCi) in a total volume of 0.075 ml. The poly(ADP-ribose) polymerase activity is shown for a 30 min incubation. Inhibitors were added to the reaction components just before addition of the cells and all inhibitors were present at 1 mM. The amount of poly(ADP-ribose) synthesized in the presence of the inhibitors is expressed as a percentage of the amount of synthesized in the control reaction. The control incubation contained 27 pmoles ADP-ribose/min/10⁶ cells. Duplicate analyses agreed within 5%.
Relation of poly(ADP-ribose) synthesis to the number of permeabilized cells in the reaction mixture. The reaction mixture contained 40 mM Tris-HCl (pH 7.8), 2 mM MgCl₂, 0.2 M sucrose, 1 mM β-mercaptoethanol, 0.8 mM EDTA, 1 mM (ade⁻¹⁴C) NAD (0.1 μCi) and the indicated number of permeabilized SVT2 cells at 5 x 10⁵ cells/cm² in a final volume of 0.1 ml. Reactions were incubated at 25°C for 10 min.
INCORPORATION OF ADP-RIBOSE
Figure 11

Effect of MgCl₂ concentration on poly(ADP-ribose) synthesis in permeabilized SVT2 cells. The reaction mixture contained 40 mM Tris-HCl (pH 7.8), 0.2 M sucrose, 1 mM β-mercapto-ethanol, 0.8 mM EDTA, 1 mM (ade⁻¹⁴C)NAD (0.1 μCi) and 3.5 x 10⁶ permeabilized cells at 5 x 10⁵ cells/cm² in a final volume of 0.1 ml. Reactions were incubated at 25°C for 10 min.
INTEGRATION OF ADP-1RIBOSE (pmoles/min/10^6 cells) vs. (MgCl₂), mM
is distinctly different in permeable cells than that of isolated cell nuclei where the polymer synthesis was quite constant from 10 mM to 30 mM concentrations of MgCl₂. The pH profile for the assay is shown in Figure 12. The optimum pH range for the reaction was between 7 and 8 pH units. For subsequent experiments, pH 7.8 was chosen as the optimum pH. The rate of poly(ADP-ribose) synthesis in permeable cells was also examined as a function of temperature (Table IX). At 25°C and 30°C, the rate of synthesis was very similar, however, at 37°C, I observed a 1.7-fold increase. The stability of the polymer synthesized at these temperatures was also examined. At lower temperatures, such as 25°C and 30°C there was no degradation of the polymer; however, at 37°C, 16% of the polymer was degraded. Thus the optimum temperature was chosen to be 25°C. At this temperature, the degradation rates of the polymer synthesized by logarithmically growing and stationary phase 3T3 cells were examined (Figure 13). The poly(ADP-ribose) synthesized in both of these situations was quite stable. In stationary phase cells, about 7% of the polymer was degraded after 30 min incubation with unlabelled NAD, whereas in logarithmically growing cells, there was no apparent degradation. The synthesis of poly(ADP-ribose) was also examined in the presence of increasing amounts of DNase I (Figure 14). Stimulation by DNase I occurred in a dose-dependent fashion up
Figure 12

Effect of pH on poly(ADP-ribose) synthesis by permeabilized 3T3 cells. The reaction mixture contained 30 mM MgCl$_2$, 0.2 M sucrose, 1 mM β-mercaptoethanol, 0.8 mM EDTA, 1 mM (ade-$^3$H) NAD (0.8 μCi), $2 \times 10^6$ permeable 3T3 cells at a density of $4.8 \times 10^4$ cells/cm$^2$ in a final volume of 0.075 ml. The permeabilization buffer was 60 mM MES (pH 6.0) or 40 mM Tris-HCl (pH 7, 8 and 9). Reactions were incubated at 25°C for 30 min.
TABLE IX
SYNTHESIS AND STABILITY OF POLY(ADP-RIbose) IN PERMEABLE 3T3 CELLS
AS A FUNCTION OF TEMPERATURE

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Poly(ADP-ribose) (pmoles ADP-ribose/10^6 cells)</th>
<th>Additional 30 min incubation with unlabelled NAD</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>237</td>
<td>230</td>
</tr>
<tr>
<td>30</td>
<td>229</td>
<td>236</td>
</tr>
<tr>
<td>37</td>
<td>289</td>
<td>243</td>
</tr>
</tbody>
</table>

Permeabilized 3T3 cells (2 x 10^6 cells) at a density of 3.5 x 10^4 cells/cm^2 were incubated with 40 mM Tris-HCl (pH 7.8), 0.2 M sucrose, 1 mM β-mercaptoethanol, 0.8 mM EDTA, 30 mM MgCl_2, 1 mM (ade-^3^H)NAD (0.8 μCi) in a final volume of 0.075 ml at 25°C.
Figure 13

Stability of poly(ADP-ribose) in permeable 3T3 cells. Log phase cells at a density of $4 \times 10^4$ cells/cm$^2$ and stationary phase cells at a density of $2.4 \times 10^5$ cells/cm$^2$ were permeabilized and $2 \times 10^7$ cells were incubated in a total volume of 1 ml with the complete poly(ADP-ribose) reaction mixture containing 1 mM (ade-$^{14}$C)NAD (1 μCi). After 30 min incubation at 25°C, cells were collected by centrifugation and resuspended in the poly(ADP-ribose) reaction mixture containing 1 mM unlabelled NAD (time zero). The cell suspensions were incubated at 25°C for an additional 60 min and 0.1 ml aliquots containing $2 \times 10^6$ cells were removed at indicated times, precipitated with 40% (W/V) TCA and processed as described in METHODS. The amount of residual radioactivity in log and stationary phase cells, measured at indicated times are expressed as percentages of the radioactivity in the respective log (●-●) or stationary phase cells (○-○).
Figure 14

Synthesis of poly(ADP-ribose) in permeabilized SVT2 cells in the presence of DNase I. The reaction mixture (0.1 ml) contained $3 \times 10^6$ permeable cells in 40 mM Tris-HCl (pH 7.8), 0.2 M sucrose, 30 mM MgCl$_2$, 1 mM β-mercaptoethanol, 0.8 mM EDTA, 1 mM (ade-$^{14}$C)NAD (0.1 μCi) and the indicated amount of DNase I. Reactions were incubated at 25°C for 10 min.
to 1 mg/ml, but at higher concentrations of DNase I, the synthesis of the polymer levelled off. I shall refer to the enzyme activity measured in the absence of DNase I as the basal enzyme activity, and the enzyme activity in the presence of saturating levels of DNase I as the maximal enzyme activity. Figure 15 compares the basal and maximal enzyme activities in permeable cells as a function of time. The synthesis of the polymer was stimulated up to 11-fold upon incubation with 1 mg/ml DNase I for 30 min. The basal enzyme activity was linear up to 50 min incubation, whereas the maximal enzyme activity was linear only for about 10 min.

The effect of various concentrations of NAD on the catalytic activity of poly(ADP-ribose) polymerase was examined. Figure 16 shows Lineweaver-Burk plots for the poly(ADP-ribose) polymerase in permeabilized 3T3 cells. The $K_m$ for NAD obtained for the basal enzyme activity was 127 μM and for the maximal enzyme activity was 119 μM. These $K_m$ values are very similar to those obtained with isolated cell nuclei.

**Poly(ADP-ribose) Polymerase Activity in Permeable Cells as a Function of Growth**

The activity of poly(ADP-ribose) polymerase in permeable normal and transformed 3T3 cells was examined as a function of growth (Figure 17). At indicated cell densities, cells were permeabilized and incubated with labelled NAD for 30 min at 25°C. The variation in poly(ADP-ribose) polymerase
Basal and maximal poly(ADP-ribose) synthesis in permeable 3T3 cells as a function of time. Log phase 3T3 cells (2 x 10^6 cells) at a density of 2 x 10^4 cells/cm^2 were permeabilized and incubated in a total volume of 0.1 ml with 10 mM Tris-HCl (pH 7.8), 0.2 M sucrose, 30 mM MgCl_2, 1 mM β-mercaptoethanol, 0.8 mM EDTA, 1 mM (ade-^{14}C)NAD (0.1 μCi) in the presence and in the absence of 1 mg/ml DNase I. Incubations were carried out at 25°C. Poly(ADP-ribose) synthesis is expressed as pmoles of (ade-^{14}C)ADP-ribose incorporated per 10^6 cells. Basal poly(ADP-ribose) synthesis, (o-o); maximal poly(ADP-ribose) synthesis, (●-●).
INTEGRATION OF ADP-RIBOSE

(pmoles / 10^6 cells) x 10^{-3}

TIME (min)
Determination of the apparent Michaelis-Menten constant.

Poly(ADP-ribose) polymerase in permeabilized 3T3 cells was assayed as described in METHODS in the presence of various concentrations of NAD and with or without 1 mg/ml DNase I. Initial velocities were determined from 4 min incubations at 25°C. The $K_m$ values for NAD were calculated by a computer program as described (193). Panel A: Basal enzyme activity; Panel B: Maximal enzyme activity.
activity in 3T3 and SVT2 cells as they grew from low to high densities is shown in Fig. 17. The basal enzyme activity in 3T3 cells decreased gradually as cells approached high densities. When these cells entered DDIG, the basal enzyme activity showed a rapid 6-fold increase. I have shown previously that degradation of poly(ADP-ribose) is negligible both in logarithmically growing and stationary phase 3T3 cells under the experimental conditions employed. Therefore, we can discard the possibility of differential degradation of the polymer having a role in the accumulation of poly(ADP-ribose) in stationary phase cells.

The basal enzyme activity in SVT2 cells showed a gradual decrease as cells reached high densities; however, it remained constant at cell densities where 3T3 cells exhibit DDIG. The maximal enzyme activity measured in the presence of 1 mg/ml DNase I remained unchanged as a function of growth and was higher than the basal activity both in 3T3 and SVT2 cells. However, the average maximal activity of poly(ADP-ribose) polymerase in 3T3 cells was twice that of the SVT2 cells.

Since permeabilized stationary phase 3T3 cells had higher levels of poly(ADP-ribose) polymerase activity than those of logarithmically growing cells, it was of interest to see if the synthesis of longer chains of the polymer in stationary phase cells could account for this increased
Variation of poly(ADP-ribose) polymerase activity with 3T3 and SVT2 cell density. Confluent dishes of cells were sub-cultured at a density of $5 \times 10^3$ cells/cm$^2$ in fresh medium containing 10% fetal calf serum at time zero. Media was replaced with fresh media every 48 hours. At indicated cell densities, cells were permeabilized and incubated for 30 min as described in METHODS. The assays for poly(ADP-ribose) synthesis at each time point were performed on $2 \times 10^6$ permeable cells in a total volume of 0.1 ml in the presence of (ade-$^{14}$C)NAD (0.1 μCi). The enzyme activities were expressed as pmoles (ade-$^{14}$C)ADP-ribose incorporated per min per $10^6$ cells. Basal poly(ADP-ribose) polymerase activity in 3T3 cells, (▲-▲); SVT2 cells, (●-●). Maximal poly(ADP-ribose) polymerase activity in 3T3 cells, (Δ-Δ); SVT2 cells, (○-○).
activity. To answer this question, the chain length of the polymer synthesized by logarithmically growing and stationary phase 3T3 cells was determined (Table X). To distinguish between elongation and initiation of polymer synthesis, chain length of the polymer was determined as a function of incubation time. As shown in Table X, the average chain length of the polymer in logarithmically growing and stationary phase cells was quite similar, and did not change as a function of incubation time. These observations suggest that initiation of new polymer chains is responsible for the observed increase in enzyme activity in stationary phase 3T3 cells.

I have also determined the average chain length of the polymer synthesized by stationary phase cells in the presence of 1 mg/ml DNase I. As shown in Table X, the average chain length of the polymer synthesized in the presence of DNase I is only 40% longer than the poly(ADP-ribose) synthesized by the stationary phase cells in the absence of DNase I. Since such a small difference in the chain length of the polymer cannot account for the 4-fold stimulation in enzyme activity observed with DNase I, I conclude that poly(ADP-ribosylation) of new sites is responsible for the enhanced enzyme activity.

In experiments previously described, I have shown that there is an increase in poly(ADP-ribose) polymerase activity in 3T3 cells grown as monolayers in which cessation of growth was caused by density-dependent inhibition of growth. My findings are in complete disagreement with those of Miwa and
**TABLE X**

CHAIN LENGTH OF POLY(ADP-RIBOSE) SYNTHESIZED BY PERMEABLE 3T3 CELLS

<table>
<thead>
<tr>
<th>Growth Phase</th>
<th>Incubation Time (min)</th>
<th>PR-AMP (% Total Counts)</th>
<th>AMP</th>
<th>Average Chain Length (PR-AMP/AMP + 1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Log</td>
<td>5</td>
<td>89.9</td>
<td>7.6</td>
<td>12.8</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>83.4</td>
<td>8.2</td>
<td>11.2</td>
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<tr>
<td></td>
<td>30</td>
<td>80.0</td>
<td>9.4</td>
<td>9.5</td>
</tr>
<tr>
<td>Stationary</td>
<td>5</td>
<td>85.6</td>
<td>9.7</td>
<td>9.8</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>86.7</td>
<td>7.7</td>
<td>12.3</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>77.6</td>
<td>6.9</td>
<td>11.2</td>
</tr>
<tr>
<td>Stationary + DNase I</td>
<td>30</td>
<td>87.7</td>
<td>6.0</td>
<td>15.6</td>
</tr>
</tbody>
</table>

Logarithmically growing (4 x 10⁴ cells/cm²) and stationary phase (1.8 x 10⁵ cells/cm²) 3T3 cells were permeabilized and incubated with 1 mM (ade⁻³H)NAD in the presence and absence of 1 mg/ml DNase I. Chain length of the polymer was determined as described in METHODS.
coworkers (120), who examined poly(ADP-ribose) synthesis in monolayer cultures of African green monkey kidney and mouse fibroblasts as a function of growth. They reported that the enzyme activity in these cells remained low throughout the growth cycle. These investigators have studied the enzyme activity in isolated cell nuclei over a six-day growth period without changing the growth medium. Thus exhaustion of the growth media might have been one of the factors that contributed to the different results. It was, therefore, of interest to answer the question of how the activity of the polymerase was being affected by the nutritional condition of the cell cultures. I designed experiments to assay the enzyme activity in cell cultures under different feeding schedules. Cell growth was monitored utilizing two parameters; cell number and the rate of DNA synthesis. As shown in Fig. 18, in cell cultures which were fed with fresh media containing 10% fetal calf serum every 24 hours, cells grew logarithmically for 4 generations and then ceased growth at about $1.8 \times 10^6$ cells/35mm dish. The rate of DNA synthesis decreased as the cells approached stationary phase of growth, showing an inverse relation to poly(ADP-ribose) polymerase activity. Figure 19 shows the results obtained with cell cultures whose growth medium was placed every 72 hours. In these cell cultures, the cells grew logarithmically for 4 generations and then ceased growth at
Poly(ADP-ribose) polymerase activity and rate of DNA synthesis in 3T3 cells as a function of growth. 3T3 cells at near confluency were seeded in 35-mm petri dishes at $5 \times 10^4$ cells/dish in 2 ml medium containing 10% fetal calf serum and cultured at 37°C. Media were replaced with fresh media every 24 hours. At the times indicated, the cells were permeabilized and the enzyme activity was assayed as described in METHODS. Cell numbers were determined with an electronic particle counter. Rates of DNA synthesis were measured by exposing dishes of cells to 1 μCi of (methyl-$^3$H)thymidine in 1 ml of medium lacking serum for 30 min. Counts in DNA were determined by counting acid-insoluble radioactivity. Cell number, (Δ-Δ), poly(ADP-ribose) polymerase activity, (●-●); rate of DNA synthesis, (○-○).
Figure 19

Poly(ADP-ribose) polymerase activity and rate of DNA synthesis in 3T3 cells as a function of growth. Cells were grown as described in the legend to Figure 18 except that media was replaced every 72 hours. The arrows indicate the times at which the media was changed. Cell number, (△-△); poly (ADP-ribose) polymerase activity, (●-●); rate of DNA synthesis, (○-○).
about $8 \times 10^5$ cells/35mm dish. Similar to the cell cultures which were fed every 24 hours, the rate of DNA synthesis decreased as the cells approached stationary phase of growth. However, upon media change, 72 hours after the last feeding time, a 5-fold increase in the rate of DNA synthesis and a 3-fold decrease in the poly(ADP-ribose) polymerase activity was observed. This suggested that replacement of the growth media with fresh media after 72 hours stimulated growth in stationary phase 3T3 cells which was reflected by the increased rate of DNA synthesis. The stimulation of cell growth was not reflected, however, by the cell numbers, probably because this point was taken prior to cell division. Taken together, the feeding experiments suggested that the nutritional condition of the cell cultures is very critical to the poly(ADP-ribose) polymerase activity and cessation of cell growth can be brought about at different cell densities by different feeding schedules.

I then wanted to answer the question as to how the activity of the polymerase was affected when the cessation of cell growth was brought about by serum starvation which can arrest growth at much lower cell densities. The answer to this question would also clarify the point whether the high poly(ADP-ribose) polymerase activity is associated with high cell density or merely with the cessation of growth. 3T3 cells were seeded at $1 \times 10^5$ cells/35mm dish
in media containing 2% serum and allowed to reach quiescence at $1.8 \times 10^5$ cells/35mm dish (Fig. 20). After 48 hours in stationary phase of growth, cell growth was stimulated by the addition of fresh media containing 5% serum. After 3 generations of growth, cells reached a second plateau at a density of about $6 \times 10^5$ cells/35mm dish. Cell growth was initiated one more time by the addition of fresh media containing 10% serum. Two days after that, cells reached stationary phase of growth at about $1.9 \times 10^6$ cells/35mm dish. The generation time for cells growing in 2%, 5% and 10% serum was 33, 27 and 17 hours, respectively. Poly(ADP-ribose) synthesis was high when cells reached quiescence, and it decreased about 3-fold upon stimulation of growth by the addition of higher concentrations of serum containing media. However, the enzyme activity in the second and third plateau was 40% and 100% higher than that of the first plateau phase. This observation suggests that the increase in poly(ADP-ribose) polymerase activity is associated with cessation of growth as well as with the increase in cell density.

**Poly(ADP-ribose) Polymerase Activity and DNA Strand Breaks**

I have presented data obtained from isolated cell nuclei and permeabilized cells which indicated that the poly(ADP-ribose) polymerase activity varies with the growth state of the cells. Table XI compares the enzyme activity
Poly(ADP-ribose) polymerase activity in serum starved 3T3 cells. 3T3 cells at near confluency were seeded at about 1 x 10^5 cells/35-mm dish in 2 ml medium containing 2% calf serum and allowed to reach quiescence. After 94 hours, growth medium was replaced with fresh media containing 5% calf serum to stimulate growth. Four days later, cell growth was stimulated once more by the addition of 10% calf serum containing media. Cell numbers and poly(ADP-ribose) polymerase activity were determined as described in the legend to Figure 18. Cell number, (Δ-Δ); poly(ADP-ribose) polymerase activity, (●-●).
in permeabilized 3T3 cells and in isolated 3T3 cell nuclei as a function of growth. Permeabilized cells exhibited a 5-fold increase in enzyme activity in stationary phase of growth, whereas the enzyme activity in isolated nuclei decreased 2.5 fold as cells ceased growth. Furthermore, the poly(ADP-ribose) polymerase activity in nuclei isolated from logarithmically growing cells was 14-fold higher than the enzyme activity observed in permeabilized cells from growing cultures. However, the enzyme activity in stationary cell cultures was very similar in permeable cells and isolated nuclei.

As indicated by the data presented before and several published reports (42,117,171), the activity of the poly(ADP-ribose) polymerase is profoundly influenced by agents which cause DNA strand breaks. Since the discrepancy observed between the two systems in terms of enzyme activity as a function of growth may be due to the presence of DNA damage produced by experimental manipulations, it was important to analyze the integrity of the DNA in both situations. Therefore, the DNA of intact cells, permeable cells and isolated nuclei were analyzed by alkaline sucrose gradients. As shown in Fig. 21, there was no detectable difference in the size of the DNA in intact and permeable cells as indicated by similar sedimentation profiles. In contrast to this situation, the size of the DNA in isolated
**TABLE XI**

POLY(ADP-RIBOSE) POLYMERASE ACTIVITY IN PERMEABLE 3T3 CELLS AND ISOLATED CELL NUCLEI AS A FUNCTION OF GROWTH

<table>
<thead>
<tr>
<th>Growth Phase</th>
<th>Poly(ADP-ribose) Polymerase Activity (pmoles/min/10^6 cells or nuclei)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Permeable Cells</td>
</tr>
<tr>
<td>Log</td>
<td>4.1</td>
</tr>
<tr>
<td>Stationary</td>
<td>18.4</td>
</tr>
</tbody>
</table>

Poly(ADP-ribose) polymerase activity in permeabilized 3T3 cells and isolated 3T3 cell nuclei was determined at indicated cell densities as described in METHODS. Incubations were carried out for 10 min at 25°C. DNA was determined by diphenylamine assay.
Figure 21

Velocity sedimentation on alkaline sucrose density gradients of intact, permeabilized 3T3 cells and isolated 3T3 cell nuclei. 3T3 cell DNA was prelabelled with (methyl-\(^{14}\)C)thymidine (0.17 \(\mu\)Ci/ml) for 22 hours and the label was chased for 1 hour as described in METHODS. About \(1 \times 10^5\) cells, permeabilized cells or isolated cell nuclei were lysed for 4 hours directly on top of the preformed gradients, and centrifuged at 12,000 RPM for 12 hours in a SW 50.1 rotor at 18\(^\circ\)C. Top of the gradient is at left, direction of sedimentation is to the right. Untreated cells, (o-o); permeabilized cells, (▲-▲); isolated nuclei, (●-●). The vertical axis shows the percentage of the total recovered counts in each fraction.
nuclei was considerably smaller, as shown by the displacement toward lower molecular weight position on the gradient. This observation indicates that the nuclei isolation procedure was producing DNA strand breaks, which in turn may be responsible for the increased levels of poly(ADP-ribose) polymerase activity. Thus, studies conducted with isolated nuclei are difficult to interpret due to the existence of endogenous DNA strand breaks which result in a high background of polymerase activity.

Although Fig. 21 shows that there is no difference between the DNA sedimentation profiles of intact and permeabilized cells, additional experiments were necessary to show conclusively whether or not DNA strand breaks were responsible for the marked increase in poly(ADP-ribose) polymerase activity observed in stationary phase permeabilized cells. I examined the DNA from logarithmically growing and stationary-phase permeabilized 3T3 cells to determine whether changes in DNA integrity occur during density inhibition of growth. Fig. 22 shows the result of an alkaline sucrose gradient performed on logarithmically growing and stationary phase permeabilized 3T3 cells pre-labelled with $^3$H-thymidine and $^{14}$C-thymidine, respectively. All of the labelled material sedimented with the characteristic of bulk cell DNA. The DNA of the logarithmically growing permeabilized cells was indistinguishable from the DNA in stationary phase permeable cells.
Figure 22

Velocity sedimentation on alkaline sucrose density gradients of permeabilized 3T3 cells. Log phase 3T3 cell (5.7 x 10^4 cells/cm^2) DNA was prelabelled with (methyl-^3H)thymidine and stationary phase 3T3 cell (2 x 10^5 cells/cm^2) DNA was prelabelled with (methyl-^14C)thymidine as described in METHODS. About 2 x 10^5 permeabilized cells were lysed at room temperature for 9 hours directly on top of the preformed gradients and centrifuged at 14,000 RPM for 12 hours in a SW 50.1 rotor at 20°C. Panel A: size of DNA in permeable log (o-o) and stationary (•-•) phase 3T3 cells. Panel B: Effect of preincubation of the permeable log (o-o) and stationary (•-•) phase 3T3 cells with poly(ADP-ribose) polymerase incubation mixture containing 1 mM NAD for 30 min at 25°C on DNA size. The total vertical axis shows the percentage of the total recovered counts in each fraction.
To examine the effect of the poly(ADP-ribose) polymerase incubation mixture on the integrity of the DNA, permeable DDIG and logarithmically growing cells were first incubated with the mixture and then subjected to alkaline sucrose gradient analysis. Fig. 22 also shows that incubation per se does not cause a change in the sedimentation profile of the DNA, suggesting that the increase in enzyme activity in permeable stationary phase cells was not associated with the development of DNA strand breaks during incubation.

Although these data suggest that the increase in poly(ADP-ribose) polymerase activity observed in permeable stationary phase cells is not due to strand breaks in DNA, the possibility still exists that the alkaline sucrose gradients were not sensitive enough to detect a small number of breaks in the DNA. This possibility was examined by the following experiment, in which DNase I was used to produce single-strand breaks in a dose dependent fashion. As shown in Fig. 23, poly(ADP-ribose) polymerase activity showed a linear increase with increasing concentrations of DNase I up to 100 μg/ml, and then reached a plateau. DNase I, at 50 μg/ml produced a 5-fold increase in enzyme activity which was equivalent to the increase observed in stationary phase permeable cells. Fig. 24 shows the sedimentation profiles obtained with DNase I-treated permeable cells when analyzed on alkaline sucrose density gradients. A dose-dependent
Figure 23

Synthesis of poly(ADP-ribose) in permeable 3T3 cells in the presence of unsaturating levels of DNase I. The reaction (0.075 ml) contained $2.2 \times 10^6$ permeable 3T3 cells at a density of $9 \times 10^4$ cells/cm$^2$ in 40 mM Tris-HCl (pH 7.8), 0.2 M sucrose, 30 mM MgCl$_2$, 1 mM β-mercaptoethanol, 0.8 mM EDTA, 1 mM (ade$^{3}$H)NAD (0.8 μCi) and the indicated amount of DNase I. Reactions were carried out at 25°C for 10 min.
POLY(ADP-RIBOSE) POLYMERASE ACTIVITY

(pmoles/min/10^6 cells)

DNase, µg/ml
Velocity sedimentation on alkaline sucrose gradients of permeable 3T3 cells treated with DNase I. About $2 \times 10^6$ log phase 3T3 cell ($9 \times 10^4$ cells/cm$^2$) DNA was prelabelled with (methyl-$^{14}$C)thymidine as described in METHODS. About $2 \times 10^6$ cells were permeabilized, incubated for 10 min with the standard poly(ADP-ribose) polymerase assay reaction mixture (0.075 ml) containing 1 mM unlabelled NAD and indicated concentrations of DNase I at 25°C. About $1 \times 10^5$ DNase I-treated permeable cells were layered on each preformed gradient, lysed for 9 hours at room temperature, and centrifuged at 14,000 RPM for 12 hours in a SW 50.1 rotor at 20°C. Radioactive counts in each fraction are expressed as percent of total radioactivity on the gradients. The arrows indicate the locations of the 16S $^3$H-labelled SV40-III, 40S $^3$H-labelled λ phage and 53S $^3$H-labelled SV40-I DNA which were utilized as DNA size markers. Top of gradient is at left, direction of sedimentation is to the right. Untreated permeable cell DNA, (o-o); treatment with 10 μg/ml DNase I, (●-●); treatment with 50 μg/ml DNase I, (Δ-Δ); treatment (o-o); with 100 μg/ml DNase I, (▲-▲).
decrease in the size of the cellular DNA was observed with increasing concentrations of DNase I.

Analysis of DNA size by this sedimentation velocity technique was sensitive enough to detect the decrease in the size of cellular DNA after treatment of permeable cells with 10 µg/ml DNase I, which resulted only in a two-fold stimulation in polymerase activity. Table XII summarizes the relationship between poly(ADP-ribose) synthesis and number of strand breaks produced by DNase I treatment of permeable cells. Calculations of number of single-strand breaks produced showed a linear relationship with the increase in enzyme activity between 0 to 100 µg/ml DNase I with approximately 300 molecules of poly(ADP-ribose) per break per 10⁶ cells. In view of these results, I concluded that the increase in poly(ADP-ribose) polymerase activity observed in stationary phase permeable 3T3 cells was not due to the production of DNA strand breaks.

**Alternate Pathways of NAD Degradation in Permeable 3T3 Cells**

In the permeabilized 3T3 cell system, I also studied the alternative pathways which utilize NAD as the substrate. The permeable cell system proves useful in the simultaneous examination of NAD degrading enzymatic activities; poly(ADP-ribose) polymerase activity can be measured in the TCA-insoluble material; whereas the TCA-soluble supernatant can
TABLE XII

RELATIONSHIP BETWEEN POLY(ADP-RIBOSE) SYNTHESIS AND NUMBER OF STRAND BREAKS PRODUCED BY DNase I

<table>
<thead>
<tr>
<th>DNase I µg/ml</th>
<th>Poly(ADP-ribose) Synthesis</th>
<th>DNA Strand Breaks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>molecules/min/cell</td>
<td>Breaks/cell</td>
</tr>
<tr>
<td></td>
<td>Increase (x 10^-6)</td>
<td>Increase (x 10^-3)</td>
</tr>
<tr>
<td>0</td>
<td>5</td>
<td>9</td>
</tr>
<tr>
<td>10</td>
<td>8.8</td>
<td>21.1</td>
</tr>
<tr>
<td>50</td>
<td>29.3</td>
<td>86.9</td>
</tr>
<tr>
<td>100</td>
<td>59.3</td>
<td>202.5</td>
</tr>
</tbody>
</table>

Poly(ADP-ribose) polymerase activity in control and DNase I treated permeable 3T3 cells was determined as described in the legend to Figure 23. Number of strand breaks in DNA produced by DNase I treatment was calculated as described in METHODS.
be assayed for the NAD glycohydrolase and NAD pyrophosphatase activities. Table XIII shows schematically the simultaneous determination of NAD-degrading enzymatic activities in permeable 3T3 cells. Permeabilized 3T3 cells were incubated in the presence of 1mM NAD containing (ade-\(^3\)H) NAD and (nicotinamide-\(^14\)C)NAD. Poly(ADP-ribose) polymerase activity was measured by the rate of conversion of \(^3\)H into the acid-insoluble material, whereas \(^{14}\)C counts in the supernatant associated with nicotinamide were a measure of total nicotinamide release. NAD glycohydrolase activity was calculated by the rate of formation of \(^{14}\)C-nicotinamide, which was corrected for nicotinamide release from poly(ADP-ribose) synthesis. NAD pyrophosphatase activity was measured in the supernatants by the rate of formation of \(^{14}\)C labelled NMN.

The effect of various concentrations of NAD on the catalytic activities of NAD glycohydrolase and NAD pyrophosphatase was examined. The Lineweaver-Burk plots for both enzymatic activities in permeable 3T3 cells is given in Fig. 25. The \(K_m\) for NAD obtained for the glycohydrolase was 102 \(\mu\)M and for the pyrophosphatase was 538 \(\mu\)M. These values are very similar to those obtained by other investigators (133,204).
TABLE XIII

SIMULTANEOUS EXAMINATION OF NAD DEGRADING ENZYMES IN PERMEABLE 3T3 CELLS

\[
\begin{align*}
\text{N*} & \quad \text{Ade}^+ \\
\text{R} & \quad \text{P} & \quad \text{P} & \quad \text{R} \\
\text{NAD} \quad \text{incubation} \\
\text{Poly(ADP-ribose)} \quad \text{NADase} & \quad \text{NAD} \\
\text{poly(ADP-ribose)} & \quad \text{nicotinamide} & \quad \text{ADP-ribose} & \quad \text{NMN} & \quad \text{AMP} \\
\text{poly(ADP-ribose)} + \text{nicotinamide} \\
\end{align*}
\]

NAD degradative activities in permeable 3T3 cells were determined simultaneously by incubation of cells with the poly(ADP-ribose) polymerase assay reaction mixture in the presence of 1 mM NAD containing (ade-\(^3\)H)NAD and (nicotinamide-\(^{14}\)C)NAD as described in METHODS. Poly(ADP-ribose) polymerase activity was measured by the rate of conversion of \(^3\)H into the acid insoluble material. NAD glycohydrolase activity was calculated from the \(^{14}\)C counts in the supernatant associated with nicotinamide which was corrected for the nicotinamide release from poly(ADP-ribose) synthesis. NAD pyrophosphatase activity was determined from the \(^{14}\)C counts in the supernatant associated with nicotinamide mononucleotide.
Figure 25

Determination of the apparent Michaelis-Menten constants for NAD glycohydrolase and NAD pyrophosphatase. Enzyme activities in permeabilized 3T3 cells from logarithmically growing cultures (7 x 10^4 cells/cm^2) were determined as described in METHODS in the presence of various concentrations of NAD. Initial velocities were determined from 4 min incubations at 25°C. The $K_m$ values for NAD were calculated by a computer program as described (193). Panel A: NAD Glycohydrolase; Panel B: NAD Pyrophosphatase.
Alternate NAD Degradative Activities In Permeable Cells As A Function of Growth

The effect of growth on poly(ADP-ribose) polymerase, NAD glycohydrolase, and NAD pyrophosphatase activities is given in Table XIV. As mentioned before, poly(ADP-ribose) polymerase activity increased 5-fold as cells entered stationary phase of growth. NAD glycohydrolase, on the other hand, showed no significant increase in activity as a function of growth. NAD pyrophosphatase activity was 5 times higher than the other two activities in logarithmically growing 3T3 cells, and increased 3-fold as cells entered stationary phase of growth.

Effect of DNA Damaging Agents on NAD Degradation

I have shown previously that agents which cause damage to cellular DNA, such as DNase I, stimulate poly(ADP-ribose) polymerase activity. I have examined the effect of DNase I on all three NAD degrading enzymatic activities. Table XV shows the relative enzymatic activities in permeable 3T3 cells in the absence and presence of DNase I. As mentioned earlier, poly(ADP-ribose) polymerase activity increased in a dose-dependent fashion, however, the NAD glycohydrolase and NAD pyrophosphatase activities remained essentially constant in the presence of DNase I. These results suggest that DNA fragmentation does not affect the activity of either NAD glycohydrolase or NAD pyrophosphatase.
TABLE XIV

POLY(ADP-RIbose) POLYMERASE, NAD GLYCOHYDROLASE AND NAD PYROPHOSPHATASE ACTIVITIES AS A FUNCTION OF GROWTH

<table>
<thead>
<tr>
<th>Growth Phase</th>
<th>Poly(ADP-ribose) polymerase activity</th>
<th>NAD glycohydrolase activity</th>
<th>NAD pyrophosphatase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pmoles NAD converted to product/min/10^6 cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LOG</td>
<td>6.0</td>
<td>23.8</td>
<td>143.7</td>
</tr>
<tr>
<td>STATIONARY</td>
<td>30.0</td>
<td>29.5</td>
<td>421.0</td>
</tr>
</tbody>
</table>

Poly(ADP-ribose) polymerase, NAD glycohydrolase and NAD pyrophosphatase activities in permeable 3T3 cells were determined by incubation of 1.8 x 10^6 logarithmically growing (5 x 10^4 cells/cm^2) and stationary phase cells (1.9 x 10^5 cells/cm^2) with 1 mM NAD containing (ade-^3H)NAD and (nicotinamide-^14C)NAD at 25^0C for 30 min. Enzyme activities are expressed as pmoles of NAD converted into product per min per 10^6 cells. Duplicate analyses agreed within 10%.
**TABLE XV**

EFFECT OF DNase I ON POLY(ADP-RIBOSE) POLYMERASE, NAD GLYCOHYDROLASE AND NAD PYROPHOSPHATASE ACTIVITIES IN NUCLEOTIDE-PERMEABLE CELLS

<table>
<thead>
<tr>
<th>DNase I (µg/ml)</th>
<th>Poly(ADP-ribose) polymerase activity</th>
<th>NAD glycohydrolase activity</th>
<th>NAD pyrophosphatase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>8.4</td>
<td>31.0</td>
<td>181.6</td>
</tr>
<tr>
<td>10</td>
<td>14.7</td>
<td>36.0</td>
<td>199.4</td>
</tr>
<tr>
<td>50</td>
<td>48.9</td>
<td>37.0</td>
<td>157.4</td>
</tr>
</tbody>
</table>

pmoles NAD converted to product/min/10^6 cells

Cultures of 3T3 cells in the logarithmic phase of growth (9 x 10^4 cells/cm^2) were permeabilized and incubated with 1 mM NAD containing (ade-^3^H)NAD and (nicotinamide-^14^C)NAD in the presence of indicated amounts of DNase I for 10 min at 25°C. Enzyme activities were determined as described in METHODS.
DNA damage can also be produced in vivo by the use of chemical carcinogens, such as N-methyl-N'nitro-N-nitroso-guanidine (MNNG). Fig. 26 shows that treatment of 3T3 cells in vivo with MNNG for 30 min prior to permeabilization results in a dose dependent ten-fold increase in the rate of poly(ADP-ribose) synthesis. The maximal poly(ADP-ribose) synthesis measured in the presence of DNase I was constant in control and MNNG-treated cells. The effect of MNNG on NAD lowering was previously studied in 3T3 cells by Jacobson et al. (92). They reported a rapid time- and dose-dependent lowering in the intracellular pools of NAD with MNNG, with no effect in the rate of biosynthesis of NAD in the presence of the carcinogen. I examined the fate of NAD in MNNG-treated 3T3 cells prior to permeabilization to characterize the pathways by which NAD was degraded. As shown in Table XVI, at 50 µg/ml MNNG, a seven-fold increase in poly(ADP-ribose) polymerase activity was observed, while this treatment did not significantly affect the NAD glycohydrolase and NAD pyrophosphatase activities. The addition of thymidine or theophylline, which are effective inhibitors of poly(ADP-ribose) polymerase activity, resulted in approximately 90% inhibition of poly(ADP-ribose) polymerase but caused less than 10% inhibition in NAD glycohydrolase and no inhibition in NAD pyrophosphatase activities. These data suggest that poly(ADP-ribose) polymerase alone is responsible for MNNG-induced degradation of NAD.
Effect of in vivo treatment of 3T3 cells with N-methyl-N'-nitro-N-nitrosoquanidine (MNNG) on poly(ADP-ribose) synthesis. Cultures of cells in the logarithmic phase of growth (5 x 10^4 cells/cm^2) were utilized. Cells were treated with MNNG for 30 min at 37°C prior to permeabilization and poly(ADP-ribose) polymerase activity at indicated MNNG concentrations was determined in the presence and absence of 1 mg/ml DNase I as described in METHODS. Enzyme activity is expressed as pmoles (ade-^{14}C)ADP-ribose incorporated per min per 10^6 cells. Enzyme activity in the absence of DNase I, (●●); enzyme activity in the presence of DNase I, (○○).
POLY(ADP-RIbose) POLYMERASE ACTIVITY

(μg/ml)
TABLE XVI

EFFECT OF MNNG ON POLY(ADP-RIBOSE) POLYMERASE, NAD GLYCOHYDROLASE AND NAD PYROPHOSPHATASE ACTIVITIES IN NUCLEOTIDE-PERMEABLE CELLS

<table>
<thead>
<tr>
<th>CELLS</th>
<th>Additions to Standard Incubation Mixture</th>
<th>Poly(ADP-ribose) polymerase activity</th>
<th>NAD glycohydrolase activity</th>
<th>NAD pyrophosphatase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pmoles NAD converted to product/min/10^6 cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>none</td>
<td>4.5</td>
<td>9.7</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>thymidine, 5 mM</td>
<td>0.2</td>
<td>9.0</td>
<td>100.8</td>
</tr>
<tr>
<td></td>
<td>theophylline, 5 mM</td>
<td>&lt;0.1</td>
<td>9.0</td>
<td>102.5</td>
</tr>
<tr>
<td>MNNG Treated</td>
<td>none</td>
<td>31.7</td>
<td>10.4</td>
<td>86.3</td>
</tr>
<tr>
<td></td>
<td>thymidine, 5 mM</td>
<td>3.3</td>
<td>9.7</td>
<td>87.5</td>
</tr>
<tr>
<td></td>
<td>theophylline, 5 mM</td>
<td>4.7</td>
<td>7.4</td>
<td>87.5</td>
</tr>
</tbody>
</table>

Cultures of 3T3 cells in the logarithmic phase of growth (7 x 10^4 cells/cm^2) were incubated for 30 min in control medium or in medium containing 50 μg/ml MNNG. Following this treatment, the medium was removed, the cells were permeabilized and incubated with 1 mM NAD containing (ade-3H)NAD and (nicotinamide-14C)NAD and enzyme activities were determined as described in METHODS. The enzyme activities shown were from progress curves constructed from samples taken at 10, 20 and 30 min of incubation at 25°C.
The main purpose of this research was to study factors which affect the rate of poly(ADP-ribose) synthesis in permeable cells. For this investigation, I utilized monolayer cultures of 3T3 cells as a model system. The advantages of such a cell system are two-fold. First, monolayer cultures of 3T3 cells maintain a well-defined regulation of growth and therefore cell growth parameters can easily be controlled. Second, the transformed counterpart cell line, which has lost the capacity to exhibit density-dependent inhibition of growth, was available.

At the time this work was initiated, the standard assay for poly(ADP-ribose) polymerase was the incubation of isolated cell nuclei with radiolabelled NAD. The necessity to utilize nuclei arose from the fact that the substrate NAD, cannot permeate intact cells. However, as shown in this study and in the study by Halldorsson et al. (75), cell manipulations inherent to nuclei isolation techniques produce DNA strand breaks, which in turn stimulate the synthesis of poly(ADP-ribose). In other words, studies which utilize isolated cell nuclei may have a high background of poly(ADP-ribose) polymerase activity, which makes interpretations with
respect to the physiological state of the cell very difficult. To avoid this problem, I adopted the cell permeabilization technique developed by Berger and Johnson (9) and modified it for its use in monolayer cell cultures. With the use of such a system I was able to measure poly(ADP-ribose) synthesis under conditions which no DNA strand breaks were detectable when analyzed on alkaline sucrose gradients. This allowed me to make careful comparisons of the poly(ADP-ribose) polymerase activity throughout the growth cycle of 3T3 cells, using methods which avoid the introduction of DNA strand breaks. Furthermore, it allowed me to correlate the number of strand breaks produced by DNase treatment of cells to the amount of poly(ADP-ribose) synthesis.

Another aspect of interest of this work was the study of the effect of agents which cause DNA damage on poly(ADP-ribose) synthesis. It was previously shown that carcinogens which are known to cause DNA damage produce a dramatic drop in the intracellular NAD levels. It was therefore important to study the degree of NAD degradation caused by enzymes which utilize NAD as the substrate. In order to have a comprehensive idea about the relationship between poly(ADP-ribose) polymerase activity and alternate NAD degrading enzymatic activities, it was necessary to develop an assay which would allow me to measure all these activities simultaneously. With the use of such an assay, I was able to
determine the relative degree of NAD degradation caused by these NAD degrading activities in cells treated with chemical carcinogens prior to permeabilization.

Characterization of the poly(ADP-ribose) polymerase activity in isolated 3T3 cell nuclei and permeabilized 3T3 cells was achieved by the use of inhibitors. My data are in good agreement with the results of others in that the most potent inhibitors are 3-aminobenzamide (49) followed by nicotinamide, thymidine, theobromine and theophylline (14,167). The effects of methylated xanthines on whole cells have generally been interpreted in terms of effects on cyclic nucleotide metabolism. Theophylline has been reported to cause an elevation in the intracellular levels of cAMP (94). Therefore, the effect of these compounds on both cAMP and NAD metabolism will have to be considered. Studies of the mode of action of cholera toxin have established a direct relationship between NAD and cAMP metabolism (64,125). The inhibition of poly(ADP-ribose) polymerase by cytokinins, as observed in the isolated cell nuclei systems, is of limited value, since these compounds were only slightly inhibitory in the permeabilized cell system. Further studies are necessary to clarify the reasons for the observed discrepancy.

The examination of poly(ADP-ribose) polymerase activity in isolated 3T3 cell nuclei and permeabilized 3T3 cells provided further insight into the properties of this enzyme.
I analyzed several parameters which affect the enzymatic activity in both systems. These include optimization of the assay conditions, stability of the product synthesized, size of the polymer chains, $K_m$ values for NAD, analysis of the DNA by velocity sedimentation on alkaline sucrose gradients and poly(ADP-ribose) polymerase activity as a function of cell growth.

The stimulatory effect of MgCl$_2$ concentration on poly(ADP-ribose) synthesis was different in permeable cells than that of isolated cell nuclei. The $K_m$ values for NAD in both systems were in good agreement with each other; this suggests that the enzyme under investigation was the same one. The differential response observed with MgCl$_2$ concentration in permeable cells versus isolated cell nuclei might be due to the differences in chromatin structure around the poly(ADP-ribose) polymerase in these two systems.

Yoshihara et al. (208), utilizing purified enzyme bound to activated DNA-cellulose, have recently shown that the self-modification of poly(ADP-ribose) polymerase is Mg$^{2+}$-dependent. In view of this observation, the higher degree of stimulation observed in poly(ADP-ribose) synthesis in permeable cells by MgCl$_2$ could be due to higher levels of auto-poly(ADP-ribosylation).

In all cases analyzed, which include normal and transformed 3T3 cell nuclei, normal permeabilized 3T3 cells and
DNase I-treated permeable 3T3 cells, the $K_m$ values for NAD were in the range of 100 $\mu$M, which is in good agreement with the values obtained by other investigators (48, 63, 130, 158, 162).

When the average chain length of the polymer synthesized by nuclei isolated from growing and stationary phase cell cultures was compared, differences in chain length were found. The polymer synthesized by the stationary phase cell nuclei had an average chain length of about 3 residues long, whereas nuclei isolated from logarithmically growing cells synthesized a polymer with an average chain length of about 4. The relationship between the average chain lengths of the polymer synthesized by nuclei isolated from growing and stationary phase cell cultures was also reflected in the enzyme activity when examined as a function of growth. The poly(ADP-ribose) polymerase activity in nuclei isolated from stationary cell cultures was $1/2$ of the activity observed in the nuclei isolated from logarithmically growing cells. As shown in Table IV, the polymer synthesized by isolated cell nuclei is very unstable. The observed reduction in polymerase activity in nuclei isolated from stationary cell cultures, as well as the reduction in the average chain length of the polymer, can therefore be partly due to the degradation of the polymer under the assay conditions employed. Recently, Miwa et al. (121) have reported that poly(ADP-ribose) synthesized by calf thymus nuclei has a branched structure with
a branching frequency of about 1 per 20-30 ADP-ribose residues. In my average chain length calculations, I have not taken into account the possible presence of the branch, and therefore, my chain length calculations would be considerably shorter, providing my isolated nuclei system also synthesizes a branched polymer. It is still possible, however, to get a general idea about the size of the polymer by the chain length calculations with the assumption that the frequency of branching is equal in the polymer synthesized by the nuclei from growing and stationary phase cell cultures.

The analyses of the DNA from isolated cell nuclei on alkaline sucrose gradients revealed that considerable DNA fragmentation takes place during nuclei isolation procedures, contributing to increased poly(ADP-ribose) synthesis. Since different nuclei isolation procedures can bring about a variable degree of DNA fragmentation, data obtained from studies which utilize isolated cell nuclei are very difficult to interpret if these studies have not examined the integrity of the DNA. In fact, there are several studies which examine poly(ADP-ribose) polymerase activity in isolated cell nuclei as a function of growth and report contradictory observations. Smulson and Rideau (172) have reported an increase in poly(ADP-ribose) polymerase activity in suspension-grown HeLa cell nuclei at a
transition from log to stationary phase, while at stationary phase cell nuclei enzyme activities were low. In contrast, Stone and Shall (176) reported a 3-fold increase in enzyme activity in isolated cell nuclei during the logarithmic phase of mouse fibroblast cell (LS cells) growth, and correlated this increase in enzyme activity with the increase in cell number. Miwa et al. (120), on the other hand, have reported that poly(ADP-ribose) polymerase activity associated with the nuclei of untransformed monolayer cultures of African green monkey kidney cells did not show a significant change during the growth cycle.

I have also examined poly(ADP-ribose) polymerase activity in isolated nuclei from transformed 3T3 cells which do not exhibit density-dependent inhibition of growth. The nuclei of transformed 3T3 cells showed no change in activity as a function of growth, and the enzyme activity in nuclei from transformed cells was similar to that of stationary phase normal 3T3 cell nuclei. The average chain length of the polymer synthesized by normal cell nuclei was slightly longer than that of the untransformed cell nuclei at corresponding cell densities; however, the average chain length of the polymer synthesized by low and high density transformed cell nuclei was very similar. Miwa et al. (120) have reported only a small difference in the chain lengths of poly(ADP-ribose) synthesized by normal and
transformed cell chromatin. In contrast to my observations, Miwa et al. (120) have reported that poly(ADP-ribose) polymerase activity associated with the nuclei is two- to ten-fold higher in transformed cells than in untransformed cells. They also reported that when confluent transformed cells were subcultured, their specific activity first decreased two- to four-fold and then rapidly increased during the exponential cell growth and slowed down when cells entered stationary phase of growth. Induction of cellular DNA synthesis in quiescent monolayer cultures of mouse fibroblasts, C3H2K-C4 cells, by either addition of fresh medium or infection with SV40 did not cause a change in enzyme activity. Since the number of cells did not increase significantly by 40 hr after infection of cells or medium change, Miwa and coworkers concluded that the increase in enzyme activity is related to the high cell density in transformed cells. In SV40tsA-transformed cells, this density-dependent increase in enzyme activity was observed when cells were cultivated at the permissive temperature. Based on these observations, Miwa and coworkers suggested that the increase in poly(ADP-ribose) polymerase activity is involved in the release of transformed cells from the density-dependent regulation of cell growth. In agreement with these results, Burzio et al. (25) reported that poly(ADP-ribose) polymerase activities in the nuclei
of Novikoff hepatoma cells and human leukemic leukocytes is higher than those in normal liver cells and leukocytes, respectively. Lehmann et al. (106) reported that nuclei extracted at sequential time intervals from mitogen-stimulated, pig lymphocytes demonstrated an increase in poly(ADP-ribose) synthesis that corresponded with the increase in DNA synthesis.

There are several factors which might have contributed to differences found in these reports. First, different results can be obtained with different cell types. I have examined the enzyme activity in the nuclei of monolayer 3T3 cell cultures, whereas other studies were conducted with nuclei isolated from suspension grown cell cultures. Second, I replaced the growth medium with fresh medium every other day, whereas Miwa and coworkers have studied the enzyme activity over a six-day growth period without changing the growth medium, allowing the cells to reach stationary phase of growth as a result of exhaustion of the growth media. Third, different nuclei isolation procedures might cause variable degree of DNA fragmentation and cause a variable degree of stimulation in poly(ADP-ribose) synthesis. Since gentle permeabilization of the cells avoids DNA fragmentation, poly(ADP-ribose) synthesis in permeable cells may more closely resemble the in vivo synthesis. With this in mind, I have utilized a permeable cell system to examine the synthesis of
poly(ADP-ribose) in normal and transformed monolayer 3T3
cell cultures as a function of growth.

When confluent dishes of 3T3 cells were subcultured,
the basal enzyme activity decreased four-fold during log-
arithmetic growth, then rapidly increased as the cells
to enter density-dependent inhibition of growth. At low
cell densities, the enzyme activity in permeabilized trans-
formed cells, however, first decreased three-fold and then
remained constant as cells entered higher densities. The
marked increase found in the synthesis of the polymer with
stationary phase normal 3T3 cells was not observed with the
transformed cell line at corresponding cell densities.
Analysis of the average chain length of the poly(ADP-ribose)
synthesized by permeable 3T3 cells as a function of growth
revealed that the average length of the polymer did not
change with the growth status of the cells. Furthermore,
synthesis of the polymer as a function of incubation time
suggested that the increase in the polymer synthesis with
time is associated with the introduction of new chains or
branches rather than the elongation of the existing unbranched
chains.

My results are in complete agreement with those of
Berger and coworkers, who examined poly(ADP-ribose) synthesis
in permeable cell systems and reported an increased poly
(ADP-ribose) polymerase activity in stationary phase mouse
L cells (11) as well as in Chinese hamster ovary (10) cells.
I have analyzed poly(ADP-ribose) synthesis in monolayer cultures of cells which exhibit density-dependent inhibition of growth in spite of daily replenishment of the growth medium. Berger and coworkers, on the other hand, have utilized suspension grown cell cultures and achieved stationary phase of cell growth by exhaustion of the nutrients in the growth media.

Although the basal poly(ADP-ribose) polymerase activity showed marked variations with 3T3 cell growth states, the total enzyme activity measured with saturating levels of DNase remained relatively constant as a function of growth in both 3T3 and SV40-transformed 3T3 cells. This suggests that the total amount of enzyme in normal and transformed cells is relatively constant and is not dependent on cell growth phase. This is an agreement with the results of Berger and coworkers, who reported that the total poly(ADP-ribose) polymerase activity in permeable mouse L cells remains relatively constant during the fluctuations in cell growth status. Berger et al. (10) also measured maximal poly(ADP-ribose) polymerase activity in permeable CHO cells synchronized by mitotic selection. They reported that the maximal enzyme activity was constant during the first 7 hr following selection. During the next 2 hr there was a 1.5-fold increase in activity. During the subsequent hour, as cells entered G2-M, the total enzyme activity decreased to its previous level and remained there for the rest of the
cell cycle. Based on these observations, they concluded that the enzyme protein may be synthesized during the end of S phase, and then the enzyme is restored to its usual level as the cells subsequently divide. I observed that the untransformed 3T3 cells had levels of maximal enzyme activity two times higher than the transformed cells. This may result from higher level of enzyme or chromosomal acceptors associated with the chromatin of 3T3 cells.

I have also examined the effect of nutritional condition of the cell cultures on poly(ADP-ribose) synthesis. Utilizing cell cultures whose media was replaced with fresh media every 24 hours or 72 hours, I have shown that the nutritional condition of the cell cultures is very critical to the measured poly(ADP-ribose) polymerase activity. Cell cultures which were fed every day exhibited density-dependent inhibition of growth at twice the cell density of those cultures which were fed every 72 hours. Furthermore, replacement of the growth medium after 72 hours caused a decrease in enzyme activity and an increase in the rate of DNA synthesis. The decrease in poly(ADP-ribose) synthesis effected by the medium change may be due to the loss of some factor(s) released into the original culture medium during the 72 hr culture or simply due to stimulation of cell growth. These observations suggested that cessation of cell growth can be brought about at different cell
densities by different feeding schedules. To examine in
more detail the relationship between the nutritional con-
dition of the cell cultures and poly(ADP-ribose) polymerase
activity, I initiated the serum-starvation experiments.

To answer the question as to whether high poly(ADP-
ribose) polymerase activity was associated with high cell
density or merely with the cessation of cell growth, I
have utilized quiescent cultures whose growth was arrested
at lower cell densities by serum starvation. I observed
increased poly(ADP-ribose) polymerase activity with quie-
scent cell cultures, whereas enzyme activity was decreased
from 5- to 7-fold upon stimulation of growth by the addi-
tion of media containing fresh serum. These results suggest
that the absolute amount of enzyme activity is associated
with cessation of growth as well as cell density, since it
is considerably higher at the 3rd stationary phase where
the cell density was the highest (Fig. 20).

Different studies suggested that poly(ADP-ribose)
synthesis either suppresses, stimulates, or has no effect
on DNA synthesis. Berger et al. (11) have reported
that suppression of DNA synthesis in LS cells due to
different treatments, such as growth to plateau phase,
shifting log phase cells to glucose deficient media, or
infection of cells with vaccinia virus was always associated
with an increase in poly(ADP-ribose) synthesis when measured
in permeable cell systems. In partial agreement with my observations, they concluded that the increase in poly(ADP-ribose) synthesis was associated with changes in DNA synthesis rather than with any change in cell density. Miller (118) has shown that treatment of HeLa cells with bleomycin, a drug which is known to cause fragmentation of the cellular DNA and inhibit DNA synthesis, results in an increased synthesis of poly(ADP-ribose). In contrast, an increase in poly(ADP-ribose) polymerase activity, along with the increase in DNA synthesis, has been reported in the nuclei of PHA stimulated pig lymphocytes (106) and in permeabilized human lymphocytes (15).

Berger et al. (12) caused the suppression of DNA synthesis by allowing wild-type CHO cells to grow to density-dependent inhibition or by shifting CS4 cells, cold-sensitive DNA arrest mutants of CHO cells, from 37°C to the non-permissive temperature of 33°C. In both cases, suppression of DNA synthesis was accompanied by an increase in poly(ADP-ribose) synthesis and by the development of DNA strand breaks, as demonstrated by the shift of a portion of high molecular weight DNA to the low molecular weight area of alkaline sucrose gradients. DNA strand breaks were also reported to occur when DNA synthesis was suppressed as part of the differentiation process. For example, induction of hemoglobin synthesis in murine erythroleukemia cells was
associated with temporary suppression of DNA synthesis, marked increase in poly(ADP-ribose) synthesis and development of DNA fragmentation (149,160,191,210). Adult rat heart cells have higher levels of poly(ADP-ribose) synthesis than neonatal rat heart cells and this increase in enzyme activity was associated with cessation of DNA synthesis and the development of increasing numbers of DNA strand breaks (35,36). I examined the DNA from logarithmically growing and stationary phase 3T3 cells by velocity sedimentation on alkaline sucrose gradients to determine whether changes in DNA integrity occurred during density-dependent inhibition of growth. All of the labelled DNA sedimented with the characteristic of bulk cell DNA (Fig. 22). The DNA of the logarithmically growing permeable 3T3 cells was indistinguishable from the DNA of stationary phase permeable cells. I have also shown that incubation of stationary phase cells with the poly(ADP-ribose) polymerase incubation mixture prior to alkaline sucrose gradient analysis does not cause the development of strand breaks in the DNA. Since my technique is sensitive enough to detect strand breaks which are responsible for only a two-fold increase in poly(ADP-ribose) synthesis as demonstrated by the DNase I experiment, I conclude that the increase in poly(ADP-ribose) activity observed in permeable stationary phase cells is not due to the development of strand breaks similar to those caused by
treatment with DNase I or by nucleases in isolated cell nuclei. We cannot rule out, however, the existence of different types of strand breaks or the existence of many nonrandom breaks on a small portion of the DNA for being responsible for the observed increase in poly(ADP-ribose) synthesis in stationary phase cells. In this context, Benjamin and Gill (8) have incubated poly(ADP-ribose) polymerase free of DNA with plasmid DNA which was fragmented with restriction enzymes, and have shown that poly(ADP-ribose) synthesis in vitro is dependent upon the number and type of strand breaks and is independent of the nucleotide sequence. They reported that double-stranded restriction fragments with flush ends are 3 times more effective than fragments with unpaired nucleotides extending from the 3' termini and 10 times more effective either than fragments with unpaired nucleotides extending from the 5' termini or than plasmids with single-strand breaks.

One of the reasons for the discrepancy observed between my results and the results of others might be the difference in the cell lines employed. Berger and coworkers conducted their experiments with CHO cells grown in suspension, whereas I utilized monolayer cultures of 3T3 mouse embryo cells which maintain a well defined regulation of growth. Secondly, we cannot exclude the possibility that the low molecular weight DNA observed by Berger and coworkers
is an artifact and represents cell death. They actually reported that when CS4 cells were returned to 37°C, they were never able to show significant growth and completely eliminate the low molecular weight DNA. These cells were also 18% trypan blue positive after 48 hrs at 37°C and this increased to 30% positive after 72 hrs at 37°C, suggesting cells were damaged beyond repair. My cell system, however, eliminates this problem since dead cells, if any, detach and they are washed off the surface of the dish before the experiment is conducted.

Analysis of the experimental results obtained from the DNase I treatment of permeable cells in vitro revealed a linear quantitative relationship between poly(ADP-ribose) synthesis and the number of DNA strand breaks produced with approximately 300 molecules of poly(ADP-ribose) per break per 10^6 cells. These results suggest that one of the factors involved in the ability of the DNA to support poly(ADP-ribose) synthesis is dependent upon the number of strand breaks it contains.

The lack of an apparent relationship between the observed increase in poly(ADP-ribose) synthesis in stationary cell cultures and DNA strand breaks may require a different explanation for the role of poly(ADP-ribose) in growth control mechanisms. I have shown that in 3T3 cells the maximal enzyme activity is quite constant as a function of cell growth. This observation suggests that
the changes in the amount of poly(ADP-ribose) synthesis in various physiological states of cells may be the result of variations in the number or availability of acceptors rather than variations in the polymer synthesizing or degrading enzymes. Several studies have reported a large number of chromosomal proteins as acceptors for poly(ADP-ribosylation) reactions. Because poly(ADP-ribose) polymerase is bound to chromatin and attaches chains of polymer to chromatin proteins (78), it has been speculated that this enzyme might form a system for the transient alteration of chromatin structure (178). Stone et al. (1978) described the formation in isolated HeLa cell nuclei of a complex histone H1 and poly(ADP-ribose) composed of two H1 molecules and one polymer chain covalently linked to one of two H1 molecules. They speculated that one function of such a complex formation can be to condense or stabilize the folds of chromatin fibers. Indeed, the amount of H1 histone-poly(ADP-ribose) complex synthesized in isolated HeLa cell nuclei has been found to correlate with the extent of chromatin condensation as effected by polyamines or divalent cations (30). Burzio et al. (28) have reported that ADP-ribosylation of H1 histone causes a conformational change which results in a diminished affinity of the histone for DNA. They hypothesized that the modification of H1 histone could diminish the crosslinking ability of the protein resulting in the relaxation of thick 250 Å chromatin
fiber to a nucleofilament of 100 Å. This could also be related to the changes in enzyme activity associated with DNA replication and DNA repair, in both of which the chromatin goes through a decondensation process. Poly(ADP-ribose) synthesis would be required for the alteration of the chromatin structure during each of these processes.

A differential pattern of protein modification by ADP-ribosylation has been observed in control cells versus cells induced to differentiate. During induction, increased poly(ADP-ribose) polymerase activity in isolated Friend cell nuclei was reported to affect mainly the modification of the histones and not that of the non-histone proteins (170). Hilz et al. (80) studied the ADP-ribosylation of nuclear proteins in many tissues at various stages of growth and differentiation. In normal and malignant hepatic tissues, NH₂OH sensitive mono(ADP-ribose) protein conjugates appeared to be associated with the growth rates. Levels of the NH₂OH resistant conjugates, on the other than, changed with the degree of differentiation.

The data available suggest a role for poly(ADP-ribose) in gene expression and its regulation. However, further studies on the individual acceptor proteins, and chain length of (ADP-ribose)_n residues in different stages of cell growth is required for a comprehensive understanding of the roles of poly(ADP-ribosylation) reactions in growth control.
The permeable cell system employed in this research proved useful in the simultaneous examination of three NAD degrading enzymatic activities. I studied poly(ADP-ribose) polymerase, NAD glycohydrolase and NAD pyrophosphatase activities in permeable 3T3 cells as a function of growth to answer the question as to how these three activities were affected by the growth stage of the cells with respect to each other. I have shown that poly(ADP-ribose) polymerase activity increases five-fold as cells enter stationary phase of growth. NAD glycohydrolase, on the other hand, did not exhibit any significant change in activity in stationary phase cell cultures and the enzymatic activity was very similar to that of poly(ADP-ribose) polymerase activity observed in nongrowing cell cultures. In logarithmically growing 3T3 cells, the NAD pyrophosphatase activity was six-fold higher than the NAD glycohydrolase activity and increased three-fold as cells entered stationary phase of growth. To my knowledge, this is the first study which examines the last two enzymatic activities as a function of cell growth. It is interesting that the NAD pyrophosphatase if the major NAD degrading activity under these assay conditions. This fact might suggest the possibility of this enzyme's being the major contributor to NAD turnover to 3T3 cells. In this context, Olivera et al. (142) have reported that in E. coli the in vivo pathway of NAD turnover involves the
initial breakage of NAD to AMP and NMN. Yamaguchi et al. (204) have also reported that the cleavage of NAD in crayfish hepatopancreas is catalyzed chiefly by a pyrophosphatase rather than by a glycohydrolase.

NAD degradation pathways have been examined by Ferro and Kun (52) in lysosome-free mitochondrial extracts from rat liver. In such extracts, they reported the existence of ADP-ribosyl transferase, NAD pyrophosphatase and NAD glycohydrolase activities. Similarly, Gill (63) analyzed soluble extracts of animal organs and identified the enzymes responsible for NAD degradation as poly(ADP-ribose) polymerase, NAD glycohydrolase and poly(ADP-ribose) phosphodiesterase which could also hydrolyze the pyrophosphate bonds of NAD. The latter enzyme has been characterized by Futai et al. (60), and was shown to be nonspecific for NAD. The $K_m$ value for NAD reported here is within the same range as those reported by others (111,204). So far, I have not studied the substrate specificity of this enzyme. Since I do not observe any degradation of poly(ADP-ribose) in my permeable cell system, I tend to believe that my NAD pyrophosphatase is not poly(ADP-ribose) phosphodiesterase. This suggestion is only tentative and further characterization of the enzyme is necessary to clarify this point.

In contrast to the studies given above, Stone and Shall (176) reported that mouse L cell nuclei do not degrade
NAD except to form poly(ADP-ribose). Wu et al. (201) have examined NAD catabolism in rabbit reticulocyte lysates and reported that NAD was rapidly and quantitatively converted to ATP and ribose-5-phosphate. The hydrolysis of NAD to ADP-ribose was catalyzed by NAD glycohydrolase and ADP-ribose was then converted to AMP by the action of ADP-ribose pyrophosphatase. ATP was formed from AMP by the action of adenylate kinase and pyruvate kinase. ADP-ribose, AMP, ADP or NMN did not accumulate in reticulocyte lysates, and the enzymatic conversion of ATP plus NMN to yield NAD was not observed. Based on these observations, they concluded that the hydrolysis of NAD to NMN by a NAD pyrophosphatase did not take place.

The increase in NAD pyrophosphatase activity observed with stationary phase 3T3 cells might suggest a role for this enzyme in growth regulation. Indeed, Anderson and Lang (4) have reported that the cleavage of NAD in mosquito was catalyzed only by the enzyme NAD pyrophosphatase, which underwent metabolic changes during different periods in the life span, and in turn the concentration of the coenzyme could be regulated by this NAD pyrophosphatase. In this context, it is important to mention that diadenosine tetraphosphate (Ap₄A), which induces replicative DNA synthesis in quiescent mammalian cells (72), is a potent inhibitor of NAD pyrophosphatase (data not shown).
Another aspect of interest in this research was to study the effect of DNA-damaging agents on NAD metabolism in 3T3 cells. The NAD degradative enzymatic activities were studied simultaneously in permeable cells after in vitro treatment of cells with different amounts of DNase I. Poly (ADP-ribose) polymerase exhibited a dose-dependent increase in activity, whereas NAD glycohydrolase and NAD pyrophosphatase activities remained unchanged in the presence and absence of DNase I. This observation suggests that the only NAD-degradative enzymatic activity affected by DNA fragmentation is poly(ADP-ribose) polymerase.

Work conducted in my laboratory indicated that MNNG causes a rapid time- and dose-dependent lowering in the intracellular levels of NAD without a significant change in the size of the NADP or ATP pools (92). Rechsteiner et al. (150) have reported that the rate of NAD turnover in mammalian cells is very rapid, with a half life of about an hour. Therefore, the effect of MNNG on NAD metabolism would involve either the inhibition of NAD biosynthesis or an increased degradation of NAD or the combination of the two. Jacobson and coworkers (92) were able to show that the presence of MNNG in the culture medium did not affect the rate of biosynthesis of NAD. This suggested that lowering of NAD must be caused by an increased rate of degradation of this nucleotide. The activities of all three NAD degrading
enzymes, poly(ADP-ribose) polymerase, NAD glycohydrolase and NAD pyrophosphatase were measured simultaneously in permeable 3T3 cells. I showed that treatment of cells with MNNG prior to permeabilization did not change the activities of the NAD glycohydrolase and NAD pyrophosphatase, but resulted in a seven-fold increase in the activity of poly(ADP-ribose) polymerase, suggesting that lowering of NAD levels is caused by enhanced synthesis of poly(ADP-ribose). This suggestion is further strengthened by the findings of Juarez-Salinas et al. (95), who observed increased in vivo levels of poly(ADP-ribose) in MNNG treated cells. My results are in agreement with those of Smulson et al. (171, 173) who have previously reported that streptozotocin and MNU treatment of HeLa cells resulted in a 1.4 and 1.8 fold stimulation, respectively, in the poly(ADP-ribose) polymerase activity in isolated cell nuclei. Similarly, Skidmore et al. (167) studied the activity of poly(ADP-ribose) polymerase in permeabilized mouse leukaemia cells, and reported that polymerase activity increases upon γ-radiation of cells in a dose dependent fashion up to 12 krad, causing a maximal 3.4-fold stimulation of the enzyme activity, whereas the degradation of NAD under conditions optimal for NAD glycohydrolase did not change. They also observed a close correlation between the increase in polymerase activity and the NAD drop following the γ-radiation. These workers made no attempt to
study the effect of $\gamma$-radiation on either NAD biosynthesis or NAD pyrophosphatase activity.

Previous studies in this laboratory indicated that theophylline, a known inhibitor of the poly(ADP-ribose) polymerase, prevents MNNG-induced lowering of NAD as well as inhibits the MNNG-induced poly(ADP-ribose) synthesis in permeable cells, and it does not affect NAD biosynthesis in intact cells (92). Therefore, the effect of poly(ADP-ribose) inhibitors on the lowering of NAD also supports a mechanism of lowering that involves increased poly(ADP-ribose) synthesis. My observations are in good agreement with those of Davies et al. (41), who reported that theophylline prevents streptozotocin-induced lowering of NAD in mouse leukemia cells, and Skidmore et al. (167), who observed inhibition of the NAD lowering caused by $\gamma$-radiation and MNU treatment of cells, by theophylline, theobromine, and 5-methylnicotinamide.

Taken as a whole, my observations suggest that carcinogen-induced lowering of NAD involves increased poly(ADP-ribose) synthesis. I have previously observed that the dose-dependent lowering of NAD is coincident with a dose-dependent appearance of alkali labile sites on DNA (92). Thus, the data presented here support the suggestion that the increased rate of poly(ADP-ribose) synthesis results from DNA damage, and the synthesis of poly(ADP-ribose) may be a requirement for DNA repair.
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