Research Overview

ADP-ribosylation of nuclear proteins

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ADP-ribosylation can be defined as the post-synthetic modification of protein by the covalent attachment of the ADP-ribose moiety of NAD. The ADP-ribosylation of elongation factor Tu is responsible for the inhibition of protein synthesis by both diphtheria and *Pseudomonas aeruginosa* toxins (Hilz & Stone, 1976). The activation of membrane adenylate cyclase by cholera toxin is thought to occur by ADP-ribosylation of the adenylate cyclase-associated GTP binding protein (Gill & Meren, 1978). It has also been proposed as a mechanism by which *Escherichia coli* RNA polymerase activity is modified during bacteriophage T4 infection (Goff, 1974; Rohrer et al., 1975; Skorko et al., 1977). Coliphage N4 has also been reported to contain an intrinsic ADP-ribosyltransferase activity (Pesce et al., 1976). ADP-ribosyltransferase activity is also present in the mitochondria and nuclei of all eukaryotic organisms examined to date.

The field of ADP-ribosylation has been comprehensively reviewed by Sugimura (1973), Hilz & Stone (1976) and Hayashi & Ueda (1977). The present review is concerned exclusively with ADP-ribosylation carried out in eukaryotic nuclei and will concentrate on the large amount of data that has emerged subsequent to the publication of other reviews.

The enzyme responsible for ADP-ribosylation in nuclei is termed poly(ADP-ribose) synthetase or polymerase; the name is derived from the fact that, unlike other ADP-ribosyl transferases, the enzyme is capable of synthesizing a protein-bound homopolymer of ADP-ribose, poly(ADP-ribose) (Chambon et al., 1966; Reeder et al., 1967; Fujimura et al., 1967). NAD is cleaved at the nicotinamide-ribose bond (bond energy ~34 kJ/mol) and the ADP-ribose moiety transferred to either a nuclear protein or a protein-bound ADP-ribose molecule. The polymer thus formed is degraded by another nuclear enzyme, poly(ADP-ribose) glycohydrolase, producing the free monomeric form of ADP-ribose (Miwa & Sugimura, 1971) (Fig. 1).

(1) ADP-ribosylation of nuclear proteins

(a) Structure of mono- and poly(ADP-ribose)-protein conjugates. ADP-ribose is attached to proteins through the free ribose molecule by two distinct types of bonds, and the observation has been made that, after incubation of isolated rat liver nuclei with NAD radioactively labelled in the adenine moiety, all radioactivity is removed by dilute alkali, but only 60% is removed by neutral hydroxylamine (Adamietz & Hilz, 1976). Endogenous mono(ADP-ribose) residues linked to proteins isolated from Ehrlich ascites-tumour cells and rat liver have also been reported to possess similar labilities (Brededorst et al., 1978c), although the relative proportions of each type of linkage are different [see Section (1)(c) below] in the two tissues. More recently, it has been suggested that a third, alkali-resistant, bond may exist *in vivo* (Adamietz et al., 1978a). It should be mentioned here that poly(ADP-ribose) is stable in alkali (Fujimura et al., 1967; Nishizuka et al., 1967).

Proposals as to the nature of the linkage to protein vary from a carboxylic acid ester (Nishizuka et al., 1968, 1969), a Schiff base with lysine (Kut et al., 1976), or a phosphodiester linkage with phosphoserine (Smith & Stocken, 1973, 1975). The carboxylic acid ester was proposed to account for the lability towards hydroxylamine. It has since been reported that glutamic acid residues at position 2 are ADP-ribosylated in both histones H1 and H2B (Burzio et al., 1979; Riquelme et al., 1979). Hayashi et al. (1975) reported poly(ADP-ribose) ribosylation at these positions and, in addition, at positions 4 and 11e in histone H1. Both groups used isolated nuclei from rat liver.

The chemical structure of poly(ADP-ribose) was first elucidated by Doly (Chambon et al., 1966). The anomeric carbon of one ADP-ribose molecule was found to be attached to the adenine moiety of the next via a 1"-2" glycosidic linkage. Subsequent n.m.r. analyses of either pure poly(ADP-ribose) (Miwa et al., 1977b) or its degradation product, 2'-5'-phosphoribosyl)-5'-AMP (Miwa et al., 1977b; Ferro & Oppenheimer, 1978; Inagaki et al., 1978), have identified the linkage as α-(1"-2"), as shown in Fig. 1.

Recent studies on the chain length of poly(ADP-ribose) formed in isolated nuclei show the product may have as many as 65 ADP-ribose residues. The earliest method of determining the chain length was by incubation of isolated nuclei with radioactively labelled NAD, digestion of the product with snake venom phosphodiesterase, resolution of 5'-AMP and phosphoribosyl-AMP by paper chromatography and analysis of the radioactivity associated with each compound. The ratio of the total radioactivity (AMP and phosphoribosyl-AMP) to the radioactive AMP gives an average chain length (Nishizuka et al., 1969). The first method of resolving poly(ADP-ribose) differing chain lengths was that of Sugimura et al. (1971). Isolated nuclei labelled with [14C]NAD were digested with Pronase and the poly(ADP-ribose) and nucleic acids were precipitated with ethanol. The pellet was resuspended and subjected to hydroxyapatite column chromatography; elution was effected with increasing concentrations of phosphate buffer. In addition to separation of nucleic acids from poly(ADP-ribose), they observed a linear relationship between chain length and phosphate concentration. The same group later analysed each of the observed peaks by gel electrophoresis and found each peak comprised two subfractions differing in both chain length and terminal structure (Tanaka et al., 1977). They suggested that the latter may be due to partial degradation by hydrolytic enzymes.

Adamietz et al. (1978b) used polyacrylamide-gel electrophoresis to separate poly(ADP-ribose) molecules of differing chain lengths after alkaline digestion of labelled isolated nuclei. Discrete bands corresponding to poly(ADP-ribose) of up to 33 residues in length were detected by fluorography. They were able to show quantitative differences in the chain-length pattern from nuclei isolated from different tissues by determining the radioactivity in each band.

A similar protocol was used by Tanaka et al. (1978) to resolve various fractions of poly(ADP-ribose) after hydroxyapatite column chromatography. They were able to show the presence of at least 65 discrete bands. After analysing the chain length of each band component by the method of Nishizuka et al. (1969), they found an increasing chain length up to (ADP-ribose)$_n$. Bands corresponding to chain lengths above this, however, showed no increased chain length by the phosphodiesterase method. They suggested that this could be due to the pres-
ence of a branched polymer having more than one AMP terminus. Recently, Miwa et al. (1979) have isolated and identified a branched structure whereby additional ADP-ribose molecules are attached to poly(ADP-ribose) via a 1'''-2'' or a 1''-3'' glycosidic linkage with the ribose to which the nicotinamide was originally attached.

Farzaneh & Pearson (1978) used the hydroxyapatite-column-chromatography method to determine the chain length of poly(ADP-ribose) during development of the toad *Xenopus laevis*. In all cases they found this method gave larger estimates than the phosphodiesterase method of Nishizuka et al. (1969). They proposed that [3H]NAD was incorporated in *vitro* on to pre-existing chains synthesized *in vivo*.

(b) Identification of proteins modified *in vitro*. Two general approaches have been adopted to identify protein acceptors *in vitro*. The first method consists of isolating specific proteins from nuclei labelled with radioactive NAD. This method is restricted to the relatively well defined proteins such as histones. In this manner several laboratories have shown histones H1 and H2B to be the major acceptors amongst the histones (see Hilz & Stone, 1976; Hayaishi & Ueda, 1977). Other acceptors that have been identified are high-mobility-group (HMG) proteins, trout specific H6 protein and protamines (Wong et al., 1977). Tanuma et al. (1977) studied the ADP-ribosylation of histones in nuclei isolated from HeLa cells. On extraction of histone H1 with HClO4 and acetic acid/urea/polyacrylamide-gel electrophoresis, the bulk of the radioactivity was seen to migrate more slowly than histone H1. Stone et al. (1977) have reported a similar result and have characterized this slower-moving material as a dimer complex consisting of two histone H1 molecules joined by a single chain of poly(ADP-ribose) of length 15 ADP-ribose units. The significance of such a modification will be discussed in Section (3)(h).

A broader study of poly(ADP-ribose)lation has been presented by Rickwood et al. (1977). [3H]ADP-ribosylated proteins were separated from nucleic acids by CsCl-equilibrium-density-gradient centrifugation, fractionated on hydroxyapatite and subjected to two-dimensional gel electrophoresis. Subsequent radioautography showed that more than 30 protein species were modified. Proteins bearing various amounts of ADP-ribose were resolved by this method.

A new approach for the separation of ADP-ribosylated proteins from unmodified proteins was recently developed by Okayama et al. (1978a). The method uses complex-formation between the cis-2',3'-dial of the 'nicotinamidé' ribose of the ADP-ribose and immobilized aminophenylboronic acid. Among the proteins shown to be modified are histones H1, H2A, H2B and high-mobility-group (HMG) proteins (Hayaishi et al., 1979), and A24 protein (Okayama & Hayaishi, 1978).

The second method used to determine if a protein is modified has been to incubate the protein in a reconstituted system with purified poly(ADP-ribose) synthetase, DNA and labelled NAD. In this manner, a (Ca2+ + Mg2+)-dependent endonuclease (Yoshihara et al., 1975), and poly(ADP-ribose) synthetase itself (Yoshihara et al., 1977) have been shown to act as acceptors. However, when Okayama et al. (1977) used histones in a similar preparation, they could not detect any radioactivity associated with any stainable protein bands after sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The bulk of the radioactivity ran with an Rf of 0.58. Ellison (1978) found the same result using a similar method; it was subsequently found that the peak was present when the enzyme preparation was omitted or when labelled NAD alone was analysed.

(c) Identification and quantification of mono- and poly-(ADP-ribosylated proteins modified *in vivo*. The identification of ADP-ribosylated proteins and the extent of their modification during various functional states of a cell is crucial to understanding the physiological significance of this modification. Clarification of these problems presents workers with several major difficulties. No suitable precursor exists for labelling ADP-ribosylated proteins *in vivo*. Use of adenine, ribose or phosphate necessitates removal of contaminants such as nucleic acids, phosphoproteins or glycoproteins. Removal must be complete, since amounts of ADP-ribose–protein conjugates appear to be much less than 1% of the amount of DNA or RNA. In addition, the lability of the linkage to proteins at neutral or alkaline pH means conditions must be carefully controlled. Also, it has been suggested that turnover is extremely rapid, possibly less than 5 min (Benjamin & Gill, 1979). Solution of these problems has been achieved by exploiting a number of properties that in combination are unique to ADP-ribose and poly(ADP-ribose). That is, they are covalently attached to protein, possess (a) pyrophosphate bond(s) and in the case of poly(ADP-ribose) contain a 1''-2'' glycosidic linkage.
ADP-ribosylation of histones has been studied by extraction with inorganic acid of cells labelled in vivo and resolving the proteins present. Ueda et al. (1975) showed that histones H1, H2 and H3 are modified, and Ord & Stocken (1975) showed that histone H1, a protein F1 and a small peptide were all modified; both rat liver and rat liver nuclei. Recently, Adamietz et al. (1978a) used a different extraction procedure to show that histone H1 was ADP-ribosylated in HeLa cells by both mono- and poly-(ADP-ribose). In contrast with the studies in vitro by Stone et al. (1977), they could not detect histone H1 dimer.

Various groups have used isotopic methods to show qualitative changes in the amount of mono- and poly-(ADP-ribose) under various cellular conditions. Colyer et al. (1973) showed differences in concentrations in poly(ADP-ribose) during the cell cycle of synchronized L-cells. After pulse-labelling with [3H]adenosine, nuclei were isolated and treated with alkali. DNA and protein were removed by high-speed centrifugation and [3H]-labelled poly(ADP-ribose) in the supernatant was recovered by acid precipitation. Poly(ADP-ribose) was then hydrolysed in HClO4 at 90°C and the radioactivity determined.

Ghani & Hollenberg (1978b) used a similar approach to show differences in poly(ADP-ribose) in chick-embryo heart cells after growth in 5% and 20% O2. Cells were labelled with [14C]ribose and treated with acid to precipitate macromolecules. After washing, the pellet was treated with alkali, digested with deoxyribonuclease and ribonuclease, followed by centrifugation and then the supernatant treated with trichloroacetic acid and washing. After treatment with acid and centrifugation again. The supernatant was freeze-dried and the radioactivity measured. When the hydrolysate was chromatographed, which showed that 11% of the recovered radioactivity corresponded to ADP-ribose and 3% to oligo(ADP-ribose). The pellet was digested with Pronase, deoxyribonuclease and ribonuclease and the products analysed by hydroxylapatite chromatography (section 1a). The material eluting at the same position as authentic poly(ADP-ribose) was hydrolysed with snake-venom phosphodiesterase. Phosphoribosyl-AMP could not be detected by t.l.c. analysis, which suggests that no poly(ADP-ribose) was present in this fraction, although it is possible that degradation occurred during the isolation procedure.

All the methods described above were used to show qualitative changes of ADP-ribosylated proteins. Described below are methods that attempt to quantify amounts in vivo.

Stone et al. (1976) used an isotope-dilution method to quantify poly(ADP-ribose) in various rat tissues. [3H]adenosine-labelled poly(ADP-ribose) of known specific radioactivity was synthesized in vitro, and then added to a homogenate of the rat tissue. After treatment with alkali and hydroxylamine the poly(ADP-ribose) was partially purified by ion-exchange chromatography. The digestion product from snake-venom phosphodiesterase, phosphoribosyl-AMP, was partially purified by paper chromatography and after digestion with alkaline phosphatase, ribosyladenosine was purified to constant specific radioactivity. From this, and the known starting specific activity, the amount of unlabelled (in vivo) poly(ADP-ribose) was calculated to be 5.59 nmol/mg of DNA for adult and 6.32 nmol/mg of DNA in neonatal-rat liver. Mono(ADP-ribose), determined by a similar method (Stone & Hiltz, 1975) gave values of 5.28 and 2.19 nmol of ADP-ribose/mg of DNA for adult and neonatal-rat liver respectively.

An optical test was developed by Goebel et al. (1977) to quantify hydroxylamine-sensitive and -resistant mono(ADP-ribosylated) proteins in rat liver and Ehrlich ascites-tumour cells. The method relies on the release of 5'-AMP, as a unique product, from ADP-ribose or mono(ADP-ribosylated) ADP-proteins upon treatment with alkali. 5'-AMP was quantified by using a linked enzyme assay. Hydroxylamine-sensitive mono(ADP-ribose) and -resistant mono(ADP-ribose) was precipitated at 1.0 mol/l of DNA and does not appear to vary throughout the growth cycle. The authors reported that amounts of poly(ADP-ribose) could be determined by the method after digestion with poly(ADP-ribose) glycohydrolase. The chain length of the polymer could also be determined by measuring the ratio of nmol of ADP-ribose released by poly(ADP-ribose) glycohydrolase to nmol of 5'-AMP released by phosphodiesterase. Although no values were reported for material in vitro, the method gave comparative values for poly(ADP-ribose) synthesized in vitro with values obtained by the method of Nishizuka et al. (1969).

Two groups have used fluorimetric assays to determine amounts of poly(ADP-ribose) in vitro. Niedergang et al. (1978) treated rat liver isolated nuclei with alkali and purified the released poly(ADP-ribose) by a four step procedure. After enzymic hydrolysis, either ADP-ribose or phosphoribosyl-AMP were quantified fluorometrically by the glyoxal method of Yuki et al. (1972). Sims et al. (1979) reported a different method based on the dihydroxyborate column of Okayama et al. (1978a). Poly(ADP-ribose) from crude cellular extracts of 3T3 cells was purified by this method, and after digestion with snake-venom phosphodiesterase and alkaline phosphate, ribosyladenosine was treated with chloroacetaldehyde. After purification by high-pressure liquid chromatography the derivative was quantified. Niedergang et al. (1978) measured values of 3.35 nmol/mg of DNA for adult rat liver. Sims et al. (1979) reported no values for the amount of poly(ADP-ribose).

Antibodies against poly(ADP-ribose) have been used as a basis for determining amounts in vivo by radioimmunoassay. The first report of antibodies raised against poly(ADP-ribose) was by Kanai et al. (1974). The final preparation was highly specific and could detect poly(ADP-ribose) at 3 μg/ml. The same authors later measured the amounts of poly(ADP-ribose) in calf thymus to be 0.02 μg/mg of DNA (Sakura et al., 1977). They also showed that anti-poly(ADP-ribose) antibodies were present in the sera of patients with the autoimmune disease systemic lupus erythematosus (Kanai et al., 1977). Okolie & Shall (1979) suggest that such antibodies may provide a useful diagnostic test for the disease. Antibodies to poly(ADP-ribose) were raised in response to injected poly(A)-poly(U) (Kanai et al., 1978) and a phosphoribosyl-AMP–bovine serum albumin conjugate (Sakura et al., 1978). No quantitative data has been reported with these antibodies. Kirilov et al. (1978) reported antibodies against poly(ADP-ribose) to evaluate amounts during the cell cycle of HeLa cells. The maximum amount was 83 ng/mg of DNA. Ferro et al. (1978) used antibodies against poly(ADP-ribose)α to determine amounts of poly(ADP-ribose) in various rat and pigeon tissues. Nuclei were isolated by an organic-solvent technique, thereby minimizing the effect of degradative enzymes. Amounts ranged from 58 ng/mg of DNA in rat liver to over 1 μg/mg of DNA in pigeon heart. It was noteworthy that treatment with nicotinamide, an inhibitor of poly(ADP-ribose) synthetase (section 1a), increased the amounts of poly(ADP-ribose) in rat liver and rat heart. This may be due to increased amounts of NAD in response to nicotinamide.

Bredehorst et al. (1978a,b) developed a radioimmunoassay based on antibodies to 5'-AMP to quantify protein-bound mono- and poly(ADP-ribose) residues. The released mono(ADP-ribose) and ADP-ribose derived from polymer, after treatment with poly(ADP-ribose) glycohydrolase, was converted into 5'-AMP by treatment with NaOH and then assayed. Values obtained for the sensitive mono(ADP-ribose) residues in stationary-phase Ehrlich ascites-tumour cells and rat liver were 0.48 nmol/mg of DNA and 5.3 nmol/mg of DNA.
respectively. Total NaOH released mono(ADP-ribose) residues were 1.66 nmol/mg of DNA for Ehrlich ascites-tumour cells and 1.26 nmol/mg of DNA for rat liver (Brederhorst et al., 1979e).

All of the methods described above depend, at some stage, on the hydrolysis of the protein–ADP-ribose bond. Desorbed below are two methods that rely on keeping this bond intact and that could thus be used for subsequent identification and quantification of modified proteins. Young & Sweeney (1978, 1979) were able to isolate ADP-ribosylated proteins from unfertilized mouse ova labelled with 3H]adenosine. Since the ova were not actively synthesizing DNA and RNA during the labelling period, all the acid-insoluble radioactive material was present as either poly(A) tracts or ADP-ribosylated proteins. These could be isolated by removal of other polynucleotides by extraction with chloroform/phenol and digestion of the precipitated material with ribonuclease followed by precipitation of the protein. Acid-soluble nucleotides and nucleosides could be removed by washing, although the authors did report that some ATP was still retained.

Whish’s group (Purnell et al., 1980; Surowy & Whish 1980) used a modification of the density-gradient-centrifugation method of Rickwood et al. (1977) to remove nucleic acids from 3H]adenosine-labelled whole-cell homogenates. The relative proportions of mono- and poly-(ADP-ribose) conjugates could be determined by t.l.c. on polyethyleneimine–cellulose after base hydrolysis. By omission of the alkali step, the proteins can be resolved by using methods used by Rickwood et al. (1977) and the 3H]ADP-ribosylated proteins detected by fluorography.

(d) Synthesis of poly(ADP-ribose). Early attempts to purify poly(ADP-ribose) synthetase were summarized by Hilz & Stone (1976), and Table 1 lists the purification procedures that have been developed since that time. Khan & Shall (1976) were the first to attempt to use affinity chromatography. A purification of 85-fold was achieved by using a column containing nicotinamide and 34-fold with one containing Blue Dextran. In both cases the yield of enzyme activity was greater than 100%. This may be due to removal of degradative enzymes or, as the authors suggest, an endogenous inhibitor. Several published methods have since used similar steps. The affinity of the enzyme for DNA was first exploited by Kristensen & Holtlund (1976). A 131-fold purification was achieved by phosphate extraction and DNA–cellulose chromatography. An additional isoelectric-focusing step was later included (Kristensen & Holtlund, 1978) to achieve a final purification of 700-fold. Tsopanakis et al. (1976) and Ellison (1978) observed that the final enzyme preparation was extremely labile. To minimize inactivation during purification, Tsopanakis et al. (1977) later used an organic solvent [95% (v/v) ethylene glycol] at 4°C and at −10°C (Tsopanakis et al., 1979a) to achieve higher purification and yield of enzyme activity.

The molecular weight of the enzyme has been shown to vary from 62 000 in pig thymus (Tsopanakis et al., 1978b) to 130 000 in calf thymus (Yoshihara et al., 1978). The most exhaustive characterization of poly(ADP-ribose) synthetase is that of Ito et al. (1979). The apparently homogeneous enzyme preparation had a mol.wt. of 110 000. Sedimentation data suggests the enzyme is a globular protein with slight asymmetry. The enzyme has a pl of 9.8 and amino acid analysis showed more lysine than arginine residues. The N-terminus appears to be blocked; this is in agreement with the data of Tsopanakis et al. (1978a) for the pig thymus enzyme.

In addition to the use of purified enzyme preparations, studies on the synthesis of poly(ADP-ribose) in isolated nuclei or crude nuclear extracts have been used to characterize poly(ADP-ribose) synthetase. The enzyme requires Mg2+ and a thiol-containing reagent for maximal activity. The pH optimum is approx. 8 and the temperature optimum is usually some 10–15°C below the optional growing temperature of the cells or tissue from which the enzyme is isolated. The $K_m$ for NAD+ varies from 40 μM in quail oviduct to 1.5 mM in mouse L-cells (Hilz & Stone, 1976). The specificity for β-NAD is high; neither NADH nor NADP+ is incorporated. Various NAD analogues with altered adenine moieties are incorporated into monomer or polymer but at much slower rates than NAD+ (Suhadoln et al., 1977). The enzyme is tightly bound to DNA, and purified preparations also have an absolute requirement for DNA (e.g. Ito et al., 1979). Fragmented DNA stimulates synthesis more than native DNA. A recent study (Benjamin & Gill, 1978) shows that plasmid-PMB9 supercoiled DNA supported activity of a partially purified enzyme preparation. Activity was further stimulated when the DNA was 'nicked' once with EcoRI restriction endonuclease or greatly stimulated when nicked over 20 times with the Haelll enzyme. Yoshihara et al. (1977), by using enzyme-saturation studies, compared the ability of various DNA molecules to support poly(ADP-ribose) synthesis. Calculations showed maximal activity at approx. 200 base-pairs with highly polymerized calf thymus DNA, 40 base-pairs with poly(dA)-poly(dT) and only 10 base-pairs with an 'active' DNA recovered during purification of the enzyme. This active DNA has been partially characterized (Yoshihara et al., 1978; Has- himoto et al., 1979). It had a mol.wt. of 200 000 and a GC content of 43%.

Histones have been reported by many groups (see, e.g., the references in Table 1) to stimulate the activity of purified poly(ADP-ribose) synthetase. Yoshihara et al. (1978) showed that although histones stimulated ADP-ribosylation 100 or 400% with intact or partially denatured calf thymus DNA respectively, activity was near maximal even without histones in the presence of poly(dA)-poly(dT) or 'active' DNA; stimulation by histones was only 8–25%. They suggest histones may stimulate

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<tr>
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<tr>
<td></td>
<td>34</td>
<td>&gt;100</td>
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synthesis by masking inhibitory sections of DNA. Okayama et al. (1977) showed that histones when added to purified enzyme and DNA were not ADP-ribosylated themselves, but activated synthesis in some other way. The same group (Ueda et al., 1979) have since shown that an ADP-ribose–histone-H1 conjugate, synthesized by the Schiff-base method of Kun et al. (1976), was able to act as an acceptor in the same system. A possible explanation for this result is that ‘initiating’ enzymes, required for attaching the first ‘ADP-ribose’ moiety to protein, are either lost or inactivated during purification of the poly(ADP-ribose) synthetase. Yoshihara et al. (1977), however, reported that a purified enzyme preparation could incorporate ADP-ribose in the presence of DNA with the enzyme itself acting as acceptor. By performing incubations with NAD for different times, they found the mobility of the enzyme on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was altered. They also were able to show, together with Nolan (1978), by using pulse–chase experiments, that elongation of poly(ADP-ribose) occurred by addition of ADP-ribose on to the AMP terminus of the growing chain. Ueda et al. (1979) reached a similar conclusion after analysing the reaction product of incubations in the presence of ADP-ribose–histone-H1 conjugates.

Synthesis of poly(ADP-ribose) in isolated nuclei is inhibited by various groups of compounds. Substrate analogues such as α-NAD and NADH are potent inhibitors (see Hilz & Stone, 1976). Poirier et al. (1978) reported that high concentrations of β-NAD, suggesting a role for co-operative systems. A product inhibition by nicotinamide and 5-methylnicotinamide has been demonstrated (eg. Clark et al., 1971). The enzyme is also inhibited by thymidine and some of its analogues (Preiss et al., 1971), although the significance of this inhibition is not understood. More recently, Levi et al. (1978) have shown that methylated xanthines and cytokinins were also good inhibitors. The most potent inhibitors are the 3-substituted benzenamides (M. R. Purnell & W. J. D. Whish, unpublished work). The latter authors also showed 3-aminoenzoic acid and various acetophenones were good inhibitors of poly(ADP-ribose) synthetase.

Polyamines have been shown to stimulate ADP-ribosylation in isolated nuclei from various tissues (Muller & Zahn, 1976a; Tanigawa et al., 1977; Perrell & Lea, 1978, 1979). A differential effect was reported in the presence and absence of Mg2+. Non-histone proteins were predominantly modified in the presence of Mg2+, whereas histones were predominantly modified in the absence of Mg2+ (Tanigawa et al., 1977). Perrell & Lea (1978) showed that spermine caused increased ADP-ribosylation of histone H1 with concomitant decreased ADP-ribosylation of core histones. These effects cannot be explained by inhibiting the enzyme, since the co-operating group (Miwa et al., 1979) showed that polyamines caused no inhibition of poly(ADP-ribose) glycohydrolase in isolated nuclei from wheat seed. Polyamines appear to cause changes in ADP-ribosylation by altering the accessibility of acceptor proteins rather than by stimulating poly(ADP-ribose) synthetase (see section 2h).

Recently two groups have developed nucleotide-permeable cell systems with which to perform poly(ADP-ribose) synthetase measurements (Halldorsson et al., 1978; Berger et al., 1978a). These authors believe that such systems more closely reflect enzyme activity in vivo than do the isolated nuclei used previously. Halldorsson et al. (1978) showed permeabilized L-cells incorporate [3H]ADP-ribose at a much lower rate than do isolated nuclei. Preincubation of permeabilized cells resulted in enzyme activity more closely resembling isolated nuclei. However, when the size of DNA was determined by alkaline sucrose density gradients, the higher activities appeared to be a result of the DNA damage. This finding was confirmed by preincubating permeable cells in the presence of the factors required for DNA repair. In this case no increase in activity was observed. Berger et al. (1978a) performed a much fuller characterization of permeable L-cells and the results obtained are reported in section (3). Perhaps most significant was the observation that added deoxyribonuclease greatly stimulated enzyme activity.

(e) Degradation of poly(ADP-ribose). Poly(ADP-ribose) has two bonds that are known to be susceptible to enzymic hydrolysis. The pyrophosphate bond is cleaved endoenzynically by a phosphodiesterase from the snake Crotalus adamanteus, producing phosphoribosyl-AMP and 5′-AMP (Chambon et al., 1966; Nishizuka et al., 1967; Fujimura et al., 1967). Exonucleolytic cleavage from the AMP terminus is mediated by a phosphodiesterase from rat liver (Futai et al., 1967, 1968; Matsubara et al., 1970). A phosphodiesterase, of unknown specificity, from cultured tobacco (Nicotiana tabacum) cells (Miwa et al., 1975a; Shinshi et al., 1976), also cleaves the polymer. However, deoxyribonuclease, ribonuclease, micrococcal nuclease, spleen deoxyribonuclease and nucleotide pyrophosphatase have no effect on poly(ADP-ribose) (Hasegawa et al., 1967).

The ribose–ribose bond is cleaved by an enzyme called poly(ADP-ribose) glycohydrolase. This enzyme was first discovered in the nuclei of calf thymus by Miwa & Sugimura (1971), who found it to be mainly associated with chromatin. It has been purified 200-fold with 5% yield and cleaves poly(ADP-ribose) exoenzymeologically, leaving the terminal ADP-ribose moiety attached to protein. The enzyme was inhibited by cyclic AMP, ADP-ribose and p-chloromercuribenzoate (Miwa et al., 1974). Ueda et al. (1972) have shown that poly(ADP-ribose) glycohydrolase occurs in a rat liver soluble fraction. This enzyme appears to be identical with enzyme found in the insoluble chromatin fraction (Miwa et al., 1972). It has also been found in mouse L-cell nuclei (Stone et al., 1973), the slime mould Physarum polycephalum (Tanaka et al., 1976), in wheat embryo (Whitby & Whish, 1978) and in rat testis (Buzzi et al., 1976). A comparison of properties is found in Tanaka et al. (1976). On the basis of different pH optima and KI stimulation there appear to be two enzymes present in rat testis. Miwa et al. (1975b) have shown that in various rat tissues poly(ADP-ribose) glycohydrolase is the major degradative enzyme for poly(ADP-ribose).

DNA has been shown to inhibit poly(ADP-ribose)glycohydrolyase in several systems (see Hilz & Stone, 1976). Denatured DNA is an extremely potent inhibitor of the enzyme. Double-stranded DNA has a variable inhibitory effect that may simply be a reflection of the single-stranded DNA impurities in the preparations used. Stone et al. (1978) showed that the enzyme was bound more tightly to denatured DNA–cellulose than to double-stranded-DNA–cellulose. Inhibition of the enzyme by high concentrations of DNA could be overcome by either increasing the ionic strength of the medium or by adding histone H1, and they suggested that both of these treatments may be displacing the enzyme from inhibitory (possibly single-stranded) portions of DNA.

Synthesis of histone H1 ‘dimer’ (Stone et al., 1977) in nuclei of various cell types showed an inverse correlation with poly(ADP-ribose) glycohydrolyase activity (Lorimer et al., 1977). Thus HeLa-cell nuclei producing histone H1 ‘dimer’ possessed no detectable poly(ADP-ribose) glycohydrolyase activity, whereas MTW-9 rat mammary nuclei had high enzyme activities but no dimer. It was shown that calf thymus poly(ADP-ribose) glycohydrolyase (purified 200-fold by the method of Miwa et al., 1975) hydrolysed histone H1 dimer from HeLa cells at one-ninth the rate of an equivalent mass of poly(ADP-ribose)1. The rate of degradation of the ‘dimer’ was increased 2-fold by the addition of DNA at a ratio of 2:1 with histone H1 (Stone et al., 1978), suggesting that DNA may cause a conformational change in the structure of the ‘dimer’.

Various workers have observed that poly(ADP-ribose) glycohydrolyse does not remove the final ADP-ribose residue from protein (Buzio et al., 1976; Miwa et al., 1974; Stone et al., 1977). More recently, Okayama et al. (1978b) have shown the presence of an enzyme in rat liver nuclei that removes the ADP-
ribose from mono(ADP-ribosyl)ated histone H2B. The reaction product is not, however, ADP-ribose.

(2) Biological role of ADP-ribosylation

(a) Introduction. Since Hogboon & Schneider (1952) discovered that NAD was pyrophosphorolytically cleaved in the nucleus, various authors have suggested that NAD fulfills a role in addition to that of respiratory coenzyme in eukaryotic cells. As early as 1958, Morton suggested that the half-life of NAD is 1h. They had pre-

be due to loss of enzyme activity as a result of increased nuclear fragility.

Since the discovery of poly(ADP-ribose) synthetase, however, its biological role remains unclear. The earliest approach to determine its function was to isolate nuclei from cells in various states and attempt to correlate the activity of the enzyme with a particular cell state. As stated previously (section 1d) the validity of enzyme-activity measurements from isolated nuclei must be treated with caution. The permeabilized-cell system might provide a more realistic model for the study of enzyme activity than the use of isolated nuclei. Another approach has been measuring ADP-ribosylation of proteins in vivo with a particular cell status. The difficulties in measuring ADP-ribosylation of proteins in vivo have been men-

(b) DNA synthesis and cellular proliferation. Since 1970, when Burzio & Koide showed that preincubation of isolated rat liver nuclei with NAD caused a decrease in incorporation of

1HdTTP into acid-insoluble material, many workers have studied the possible involvement of ADP-ribosylation in the regulation of DNA synthesis. The results obtained show that ADP-ribosylation in isolated nuclei either decreases, has no effect or increases dTTP incorporation into DNA [for a sum-

mary, see Hilt & Stone (1976) and Hayashi & Ueda (1977)].

Since that time, Janakidevi (1978) has shown that removal of lysine-rich histones or treatment with heparin increases DNA

synthesis in isolated nuclei from pig aorta. The decrease in poly(ADP-ribose) synthesis observed with lysine-rich histone is explained by co-extraction of poly(ADP-ribose) synthetase, and they conclude that removal of lysine-rich histones or poly(ADP-ribose) synthetase exposes initiation sites for DNA synthesis. Tanigawa’s group (see references below) have shown that pre-

incubation of isolated nuclei from chick-embryo liver with NAD increased dTTP incorporation into DNA. Conversely, preincu-

bation with NAD of nuclei from hen liver decreased dTTP incorporation. Extraction of NAD-treated embryonic and adult liver nuclei with 0.35 m-NaCl with subsequent reconstitution showed that the factors responsible for suppression or stimula-

tion of DNA synthesis were present in the 0.35 m-NaCl extract (Tanigawa et al., 1978). They showed that the stimula-

tion of DNA synthesis observed with chick-embryo nuclei was due to increased accessibility of the DNA to nuclease (Tan-

igawa et al., 1978b). Administration of glucocorticoid hormone to chick embryos caused decreases in both DNA and poly(ADP-ribose) synthesis (Kitamura et al., 1979). This may be due to loss of enzyme activity as a result of increased nuclear fragility.

Miwa et al. (1977a) showed that poly(ADP-ribose) synthetase activity was 2–10-fold higher in nuclei from SV40-virus-transformed cells compared with untransformed cells. They also demonstrated that, in contrast with untransformed cells (where enzyme activity remained constant), the activity in transformed cells increased markedly throughout the growth cycle. Muller et al. (1979) found that alteration of DNA synthesis by HS (herpes simplex)-virus infection was not accompanied by a change in poly(ADP-ribose) synthetase activity in BHK cells, suggesting that ADP ribosylation plays no role in the control mechanisms for cellular or HS-virus DNA synthesis.

Ghani & Hollenberg (1978a) demonstrated that chick embryo heart cells exhibited higher poly(ADP-ribose) synthetase activity in isolated nuclei from cells grown in 5% (v/v) O2 than from cells grown in 20% O2. They also showed that the stimula-

tion of DNA synthesis was observed with lymphocyte nuclei from pig aorta. The decrease in DNA synthesis in isolated nuclei from pig aorta when simultaneous synthesis of both polymers occurred, the redox potential of cellular proliferation must be treated with reservation, since all are known to affect cellular processes other than ADP-ribosylation. It is considered that the use of 3-aminobenzamide and especially 3-methoxy-

benzamide will be extremely useful in this context.

A major problem in ascribing a precise role for ADP-ribosyla-

tion has been the difficulty in correlating the data obtained by the above methods with a defined cell function; e.g., during dif-

ferentiation of a particular cell, the rate of DNA synthesis will alter, the patterns of DNA transcription will change and the cell may enter a different stage of the cell cycle.

For these and other reasons, attempts to elucidate the bio-

logical role of ADP-ribosylation have produced wide-ranging and contradictory suggestions for its function. Therefore, with these reservations in mind, the various cellular functions that have been ascribed to poly(ADP-ribose) are presented below.

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Since that time, Janakidevi (1978) has shown that removal of lysine-rich histones or treatment with heparin increases DNA
they were able to show that decreased DNA synthesis in cells subjected to acute glucose deficiency, vaccinia virus infection and cytotoxic arabinoside treatment, resulted in increased poly(ADP-ribose) synthetase activity in permeabilized cells (Berger et al., 1978a,b).

When normal and chronic lymphocytic leukaemia (CLL) lymphocytes were examined with respect to phosophoamiglutinin stimulation (Berger et al., 1978d), differences were observed. In both normal and CLL lymphocytes phosophoamiglutinin stimulation caused increased ADP-ribosylation. The normal cells showed the expected response of DNA synthesis to phosophoamiglutinin stimulation, but the response of DNA synthesis in the CLL cells was decreased and much delayed. The authors suggested that this may be due to either damaged or disordered DNA (section 1d) or the presence of immature differentiating lymphocytes.

Finally, Hilz's group (R. Bredehorst, M. Goebel, F. Renzi, M. Kittler, K. Klapproth & H. Hilz, unpublished work) have examined the activity of poly(ADP-ribose) synthetase in permeabilized cells and the concentrations in vivo of mono(ADP-ribose)lated proteins throughout the growth cycle of Ehrlich ascites-tumour cells. They showed that transition from exponential to stationary phase was associated with an increase in both intrinsic poly(ADP-ribose) synthetase activity and cytidylyamine-resistant mono(ADP-ribose)lated proteins. The total poly(ADP-ribose) synthetase activity and cytidylyamine-sensitive mono(ADP-ribose)lated proteins remained unchanged, however, when the cells were made to provide the basic model for the role of ADP-ribosylation in DNA synthesis by incubating tissue slices with NAD⁺ (Claycomb, 1976a). However, results from such experiments are impossible to interpret, since NAD⁺ does not cross the cell membrane. It is noteworthy that NAD⁺ stimulates DNA synthesis in human bone-marrow cells. The authors suggested that this may be due to an exogenous enzyme system (Schacter & Burke, 1978).

(c) DNA transcription. The observation that differentiated rat liver cells engaged in RNA synthesis possessed a higher activity of poly(ADP-ribose) synthetase than cells engaged primarily in DNA synthesis (Haines et al., 1969) was the first observation suggesting a possible role for ADP-ribosylation in DNA transcription. Since then, several reports have emerged that agree or conflict with such a role for ADP-ribosylation. One approach that has been made to clarify this situation has been to correlate poly(ADP-ribose) synthetase activity with the RNA-synthesizing capacity in various systems. Thus it was shown that there was no change in poly(ADP-ribose) synthetase activity in livers from sham-operated, adrenalectomized and cortisol-treated rats (Hilz & Kittler, 1971), suggesting no role in DNA transcription. However, observation that the specific activity of poly(ADP-ribose) synthetase in nuclei and nucleoli isolated from Tetrahymena is the same, despite the high ribosomal transcriptional activity in the nucleoli, suggests no direct involvement of ADP-ribosylation in transcription. Furthermore, in the presence of active RNA synthesis, the poly(ADP-ribose) synthetase activity in both nuclei and nucleoli was the same as in the absence of RNA synthesis (Tsapanakis et al., 1978b). The involvement of ADP-ribosylation in transcription has been implicated from the results of Muller et al. (1974). These workers made the observation that, during gene expression in vivo initiated by oestrogen treatment of immature quails, an increase in RNA polymerases I and II activities is accompanied by a decrease in poly(ADP-ribose) synthetase activity in the oviduct. A possible association of poly(ADP-ribose) synthetase activity with transcription has been suggested from the studies of Smulson and co-workers, who have fractionated sonicated HeLa-cell chromatin by using ECTHAM-cellulose chromatography and glycerol-gradient centrifugation in an attempt to separate transcriptionally active and inactive chromatin (Mullins et al., 1977). These authors concluded that the poly(ADP-ribose) synthetase activity is primarily associated with extended transcriptionally active chromatin; the transcriptionally inactive, condensed, chromatin fractions contained relatively low poly(ADP-ribose) synthetase activity. This conclusion has been questioned by Yukioka et al. (1978), who have pointed out that the chromatin-fractionation technique using mechanical shearing, as used by Mullins et al. (1977), is not adequate for the separation of transcriptionally active and inactive chromatin fractions and also that sonication causes drastic changes in chromatin structure (see Yukioka et al. (1978) for chromatin-preparation references). Rat liver chromatin has been fractionated by using selective shearing with deoxyribonuclease I followed by preferential precipitation, which has been shown to fractionate chromatin into transcriptionally active and inactive fractions (Gottesfeld et al., 1974, 1975), and it was shown that poly(ADP-ribose) synthetase activity is not preferentially localized in transcriptionally active chromatin regions (Yukioka et al., 1978).

A second approach has been to look at the effect of poly(ADP-ribosylation) in isolated nuclei on subsequent RNA transcription. Thus formation of poly(ADP-ribose) in isolated rat liver nuclei did not affect the subsequent RNA synthesis by the nuclei (Burzio & Koide, 1971). Muller et al. (1974) have shown that, after preincubation of quail oviduct nuclei with NAD⁺, the activity of endogenous RNA polymerase I decreased, whereas the activity of RNA polymerase II as well as exogenous bacterial RNA polymerase remained unaffected by the ADP-ribosylation reaction. The data was suggestive of a direct ADP-ribosylation of RNA polymerase I. The amount of poly(ADP-ribose) associated with RNA polymerase I isolated from nuclei of oviducts undergoing increased transcription was seen to be lower than that associated with the RNA polymerase I from nuclei of oviducts with moderate transcription (Muller & Zahn, 1976b). More recently it has been demonstrated that preincubation of BHK-21 cells with NAD⁺ resulted in an inhibition of RNA polymerase I, and it was suggested that the inhibition was due to ADP-ribosylation of the RNA-polymerase itself, as evidenced by an inhibition of RNA polymerase activity when extracted and assayed from nuclei previously incubated with NAD⁺ (Furneaux & Pearson, 1978).

It has been shown that protein A24, which is composed of histone H2A and ubiquitin, a non-histone protein linked by an isopeptide bond (Goldknopf & Busch, 1977) is ADP-ribosylated in isolated rat liver nuclei (Okayama & Hayashi, 1978). In view of the possible role of protein A24 as a repressor of ribosomal gene activity (Ballal & Busch, 1973; Ballal et al., 1974, 1975), it has been suggested that ADP-ribosylation of this protein may be involved in the regulation of its repressor activity (Okayama & Hayashi, 1978).

(d) DNA repair. So far as a possible involvement of ADP-ribosylation in DNA repair have been based on two observations. First, it has long been known that alkylating agents and other treatments known to damage DNA result in depletion of intracellular NAD⁺ (Roit, 1956) and, secondly, that damaged DNA stimulates the activity of poly(ADP-ribose) synthetase (see section 1d).

It was shown by Smulson et al. (1975) and Whish et al. (1975) that isolated nuclei, from HeLa cells and the slime-mould Physarum polycephalum respectively, possessed higher poly(ADP-ribose) synthetase activity after pretreatment with streptozotocin, the 2-deoxy-D-glucose derivative of the alkylating agent, N-methyl-N-nitrosourea. Jacobson (1978) examined the effect, on NAD⁺ concentrations, of N-nitroso compounds that were direct-acting, indirect-acting or non-carcinogens in both 3T3 cells and mitogen-stimulated human lymphocytes. They observed large decreases in NAD⁺, and in both cell types treated with direct-acting carcinogens, a large decrease in lymphocytes, but not in 3T3 cells treated with indirect-acting carcinogens, whereas non-carcinogens had no effect on NAD⁺ concentrations in either cell type. Sudhakar et al. (1979a) showed that the N-methyl-N-nitro-
source-induced increase in poly(ADP-ribose) synthetase at the nucleosome level was a result of increased availability of protein acceptors. In a subsequent paper (Sukhakar et al., 1979b), they compared alkylation of chromatin by N-methyl-N-nitrosourea and 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea. In contrast to N-methyl-N-nitrosourea, treatment of HeLa cells with 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea resulted in a slight decrease in poly(ADP-ribose) synthetase activity in isolated nuclei. They were able to show, by nuclease digestion, that the differential effects of the two compounds on poly(ADP-ribose) synthetase may be explained by the sites of alkylation of the two compounds. N-methyl-N-nitrosourea preferentially alkylates the internucleosomal regions of DNA, which have been suggested as the sites of the enzyme binding (Mullins et al., 1978), whereas 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea alkylates the core particles.

Other treatments known to damage DNA have also been shown to stimulate poly(ADP-ribose) synthetase activity in isolated nuclei. Davies et al. (1976, 1977) have demonstrated increased enzyme activity in cells treated with γ-irradiation and induced deficiency of nicotinamide or theophylline, sufficient to prevent streptozotocin-induced decreases in NAD+ in L-cells, increased the cytotoxicity of NAD+ and reduced poly(ADP-ribose) synthetase activity as measured in isolated nuclei.

An interesting observation is that compounds such as theophylline and caffeine, known inhibitors of DNA repair (Cleaver & Thomas, 1969; Lehmann & Kirk-Bell, 1972), are also inhibitors of poly(ADP-ribose) synthetase in isolated nuclei (Levi et al., 1978). It was therefore of interest to examine the effect of other inhibitory effects of poly(ADP-ribose) synthetase on the ability of cells to repair DNA damage in vivo and their subsequent survival. The earliest study was performed by Smulson et al. (1977). At 2 days after injection of mice with LS1210 tumour cells, pretreated with or without U.V. irradiation, they were further able to show that the DNA synthesis observed after irradiation consisted of repair synthesis and not semi-conservative replication.

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Finally, it has been shown that 3T3 cells depleted of NAD+ by culture in nicotinamide-free medium were unable to undergo carcinogen-induced unscheduled DNA synthesis (Jacobson & Narasimhan, 1979). All these results suggest that ADP-ribosylation of nuclear proteins plays some, as yet unknown, role in the repair of DNA.

(e) Cell cycle. Many workers have investigated the possible involvement of ADP-ribosylation of nuclear proteins in the timing of events during the cell cycle. Before 1976, all studies were performed by isolating nuclei from synchronized cells and determining the enzyme activity. Although no consistent picture emerged, most results suggested that poly(ADP-ribose) synthetase activity was lowest in S-phase and highest in either G1- or G2-phase (Hilz & Stone, 1976). Tanuma et al. (1978) used intact and disrupted nuclei and a crude poly(ADP-ribose) synthetase preparation to measure the activity of the enzyme during the cell cycle of HeLa S3 cells. They showed that the activity of the enzyme in isolated nuclei was higher in the G2-phase, whereas in disrupted nuclei the activity increased during G2-phase, remained elevated during G2-phase and decreased after mitosis. By using crude enzyme they found a pattern similar to that obtained with disrupted nuclei. By using colcemid, a mitotic inhibitor, they found that mitotic cells had five times the activity of asynchronous cells as measured by the isolated-nuclei system. From this and previous data it is clear that isolated nuclei are unsuitable for elucidating the role of ADP-ribosylation during the cell cycle.

Berger et al. (1978b) showed that, after treatment of LS1210 cells with cytosine arabinoside (which causes accumulation of cells in G2-phase), the activity of poly(ADP-ribose) synthetase increased in permeabilized cells. By using Chinese-hamster ovary cells synchronized by micromanipulation they showed that the activity of the enzyme increased during G2-phase, decreased rapidly as the cells traversed S-phase and increased during G1-, M- and G2-phases (Berger et al., 1978c). By measuring the activity in permeabilized cells after deoxyribonuclease treatment, which was suggested to indicate total enzyme activity, they showed the activity was constant during the cell cycle except for a small peak during late S-phase, which subsequently decreases during the G1- and M-phases. They suggested that the enzyme was synthesized during S-phase and then returned to normal amounts after mitosis. This observation may account for the maxima observed during S-phase observed by other workers using isolated nuclei (Roberts et al., 1973; Colyer et al., 1973).

Barnwell & Mage (1976) used a radioimmunoassay to detect changes in poly(ADP-ribose) concentrations in HeLa cells during the cell cycle. They observed a 6-fold increase in poly(ADP-ribose) as the cells traversed from early S- to late S-phase, followed by a rapid decrease. This was followed by a 10-fold increase during G1-phase; this increase was correlated with increased poly(ADP-ribose) synthetase activity as measured in isolated nuclei. As the authors state, more information is required on the specificity of the antibody used, which Hilz's group (Wielckens et al., 1979) used an antibody to 5'-AMP to measure hydroxylamine-sensitive and -resistant mono(ADP-ribose)-protein conjugates during the cell cycle of the naturally synchronous slime mould Physarum polycephalum. They observed that the total mono(ADP-ribose) decreased from mitosis through S-phase (G1-phase is absent) and increased during G2- to M-phase. The system was analysed for hydroxylamine-lability they found different patterns for hydroxylamine-sensitive and -resistant mono(ADP-ribose)-protein conjugates. Amounts of hydroxylamine-sensitive residues decreased sharply after mitosis, remained low during S-phase and increased at the S-phase/G1-phase boundary and then remained at that value until mid-G2-phase; they then decreased during late G2-phase and rapidly increased before mitosis. Amounts of hydroxylamine-resistant residues decreased after mitosis but increased linearly during S-phase; from the S-phase/G1-phase boundary they remained constant until mid-G2-phase and then increased to pre-mitotic values. These data suggest ADP-ribosylation may fulfill more than one role during the cell cycle.

Caplan et al. (1978) used partially hepatectomized rat liver to investigate changes in ADP-ribosylation in isolated nuclei after transition from G2- to S-phase. They could detect a slight increase in ADP-ribosylation but could detect no change in the proteins released after digestions with nuclease. Since synchrony is only 30%, the methods may not be sufficiently sensitive to detect changes.

(f) Cellular differentiation and development. Caplan & Rosenberg (1975) looked at the differentiation of mesodermal cells of embryonic-chick limb buds into either muscle or cartilage, and their results were the first to suggest a possible involvement of ADP-ribosylation in differentiation. Previous work from Caplan's laboratory had shown a correlation between pyridine nucleotide concentrations and cellular differentiation, and they suggested that fluctuations in cellular NAD+ concentrations might play a role in the control of muscle as against cartilage development [see Caplan & Rosenberg (1975) for references]. The authors showed that 3-acetylpyridine potentiated cartilage differentiation that was associated with a stimulation of the rate of poly(ADP-ribose) synthesis. Administration of 3-acetylpyridine had previously been shown to decrease NAD concentrations, and the suggested mechanism was that such a change in
NAD\(^+\) concentration is 'sensed' and transposed into differential rates of ADP-ribosylation that are correlated with differentiation into either muscle or cartilage (Caplan & Rosenberg, 1975). This proposed role of 3-acytlypyridine in repressing myogenesis and enhancing chondrogenesis, however, has been questioned by McLachlan et al. (1976), who made a histological analysis of developing limb buds at various stages. They showed that the effect of 3-acetylpyridine was mediated via a destruction of the peripheral nerves, resulting in a total loss at 24 h, as well as a deleterious effect on all cell types, including cartilage, tendon, mesenchyma and muscle. Such results suggest that 3-acetylpyridine affects muscle growth rather than muscle differentiation, as does the observation that 3-acetylpyridine still has an effect on muscle tissue at stages when cartilage and muscle cells are physically separated (McLachlan et al., 1976).

Since the first observation of Caplan & Rosenberg (1975), several reports have appeared that may suggest an involvement of poly(ADP-ribose) in differentiation. For example, during the early stages of oocyte maturation in the toad Xenopus, which is mediated by progesterone, the oocyte nucleus breaks down and this is followed by a dramatic condensation of the chromosomes. It has been shown that, during such oocyte maturation, poly(ADP-ribose) synthetase activity in isolated nuclei increases 3-fold, this dramatic increase occurring before breakdown of the oocyte nucleus (Burrzo & Koide, 1977). Such results suggest a possible role for ADP-ribosylation in the early differentiation of Xenopus, and the observation (Farzaneh & Pearson, 1977) that the activity of poly(ADP-ribose) synthetase increases markedly during its embryonic development is consistent with this possibility.

Young & Sweeney (1978) have studied the incorporation of \(^{3}H\)adenosine into RNA and ADP-ribosylated protein in mouse ova and have shown that the total incorporation is maximal at 3-5 h after ovulation, which is the time of normal fertilization. The incorporation of \(^{3}H\)adenosine is low at 7-8 h, at which time the ability of the ovum to be fertilized is decreased; this suggests a possible role between adenosine metabolism, mediated in part through poly(ADP-ribosylation), and post-ovulation maturation of ova and/or their fertilizability. The above authors have also shown that mouse one-cell embryos incorporate adenosine into ADP-ribosylated material and they found that the average chain length of the polymer was 2 (a dimer) (Young & Sweeney, 1979) compared with the pentamer and monomer seen in the ovum (Young & Sweeney, 1978). In view of the apparent difference in lability of the bond linking the dimer to its acceptor in the embryo compared with the pentamer and monomer in the ovum, it was proposed that the dimer was synthesized after fertilization (Young & Sweeney, 1979). Such differential ADP-ribosylation in the ovum and embryo may indicate that a differentiation-related programme of poly(ADP-ribose) synthesis may be taking place in this system.

It has been shown by Yamada et al. (1978) that mouse myeloid leukemic cells can be induced to differentiate into cells with phagocytic activity, Fc receptors and lysosome activity, by the addition of poly(ADP-ribose) to the culture medium and that a certain proportion of these cells differentiate further into granulocytes and macrophages. Addition of dextan sulphate or poly(vinyl sulphate) was also effective in the induction of phagocytic cells and so the physiological significance of the observed stimulation of differentiation by poly(ADP-ribose) must remain questionable, although the radioactivity derived from polymer does enter the cells and becomes located in the nucleus and nuclear membrane as seen by radioautography.

Two reports have appeared that have investigated the possible role of poly(ADP-ribosyl)ation in the differentiation of erythroleukemic mouse spleen cells (Friend cells) grown in culture. Rastl & Swetly (1976), using cell line F4N, which is reported to be very sensitive to butyrate-induced differentiation and relatively insensitive to hexamethylenbisacetamide (Morioka et al., 1979), showed that using such compounds to induce differentiation resulted in a 2-4-fold increase in poly(ADP-ribose) synthetase activity as well as a concomitant transition of cells to the G\(_{1}\)-phase of the cell cycle. Morioka et al. (1979), using cell line 745, showed that poly(ADP-ribose) synthesis suppression is not inducible in the early exponential phase growth stage (14-24 h) after treatment with hexamethylenbisacetamide or dimethyl sulphoxide. With butyrate, a transient increase in ADP-riboylation in the early stages of cell growth was seen, but by 48-72 h ADP-riboylation was at the same low rate as for hexamethylendisacetamide and dimethyl sulphoxide. Nicotinamide, too, induced differentiation and also enhanced dimethyl sulphoxide and hexamethylenbisacetamide-induced differentiation and tended to inhibit butyrate-induced differentiation. Friend-cell variants unresponsive to hexamethylendisacetamide and dimethyl sulphoxide did not exhibit as low an activity of poly(ADP-ribose) synthetase as was found with normal responsive cells treated with inducers. The effect of the inducers was not mediated by a change in cell growth rate or by an effect on polygon degradation, and the results suggest that the amount of ADP-riboylation is correlated with the differentiation of the Friend cell (Morioka et al., 1979). The reason for this discrepancy between the observations of Morioka et al. (1979) and Rastl & Swetly (1978) remains to be solved, but it could be related to differences in the strain of cells used and/or differences in the culture methods. For example, Morioka et al. (1979) cultured their cells in the presence of inducers without medium change, whereas Rastl & Swetly (1978) added fresh medium to their cultures daily. Indeed Rastl & Swetly (1978) did show that the amount of poly(ADP-ribose) synthesis depended on the culture conditions.

The activity of poly(ADP-ribose) synthetase in isolated nuclei and the intracellular NAD\(^+\) concentration have been measured in differentiating rat cardiac muscle. NAD\(^+\) concentrations increase during postnatal development and does poly(ADP-ribose) synthetase activity (Claycomb, 1976b). It was suggested that the initial increase in these two parameters may be specifically related to the 30-40-fold decrease in DNA synthesis observed during this initial period and that the further increase after the second week may be concerned with terminal cell differentiation.

(g) Other possible functions of ADP-ribosylation. In addition to the above-stated postulated roles, three other possible functions have been suggested:

(i) The observation that nicotiniumadine, 5-methyl nicotinadine, thymidline and 3-isobuty1-1-methylxanthine induce ornithine decarboxylase (EC 4.1.1.17) activity prompted Minaga et al. (1978) to suggest that ADP-ribosylation may be involved in the regulation of this enzyme. Ornithine decarboxylase (EC 4.1.1.17) activity is inversely related to the 30-40-fold decrease in DNA synthesis observed during this initial period and that the further increase after the second week may be concerned with terminal cell differentiation.

(ii) Suzuki & Murachi (1978) have shown that a nucleic acid-like inhibitor co-extracts with a chromatin-bound neutral protein from rat peritoneal macrophages. They found that inhibition of proteinase activity could be removed by preincubating the de-proteinized inhibitor fraction with a crude poly(ADP-ribose) glycohydrolase preparation from rat liver before addition of the assay mixture. Deoxycybinucleoside, 1, P1 nucleoside and snake-venom phosphodiesterase has no effect. Thus they suggest that the inhibitor was similar to, but not identical with, poly(ADP-ribose). It was also shown that poly(ADP-ribose) with an average chain length of 30 residues was inhibitory.

(iii) Matinyan & Umanskii (1978) have shown that the 'poly-peptide synthetase' activity of rat liver chromatin was enhanced by preincubation with NAD\(^+\). This enhancement was inhibited.
by the presence of nicotinamide or thymidine. On storage of chromatin for 20h at 4°C, a complete loss of polypeptide syn-
thesase activity was observed. This, however, could be prevented by the presence of 3’5’-cyclic AMP, an inhibitor of poly(ADP-
ribose) glycohydrolase. They suggest that poly(ADP-ribose) may serve as an energy source for amino acid activation.

(h) Mechanisms by which ADP-ribosylation may affect cellu lar processes. The possible mechanisms by which ADP-
ribosylation may affect cellular function are:

(i) Direct modulation of enzyme activity as proposed by Yoshihara et al. (1975) and Muller & Zahn (1976b) for (Cytosine)-dependent endonuclease and RNA polymerase respectively.

(ii) Modification of a regulatory protein such as protein A24 (Okayama & Hayashi, 1978).

(iii) Alteration of chromatin structure.

Investigation of the first two possibilities awaits the identifi-
cation and elucidation of the biological role of these proteins ADP-ribosylated in vivo. Mediation by changes in chromatin
structure could occur at two levels. Firstly, it may allow
enzymes, such as DNA-repairing enzymes, access to previously
shielded DNA. The second possible role may be related to
altering gross chromatin structure, such as occurs during
chromosome condensation before mitosis. The very low con-
centration in vivo (see section 1c) would seem to preclude
involvement of poly(ADP-ribose) as a major structural ele-
ment, but may indicate that it acts as a signal to other enzymes
or proteins. Data attempting to correlate poly(ADP-ribose) syn-
thesis and chromatin structures are presented below.

It is well known that the basic structural unit of chromatin, the ‘nucleosome’, is composed of 140 base-pairs of DNA wound
around an octamer of core histones (H2B, H2A, H3 and H4). Such core particles are joined by a ‘linker’ region of DNA,
which is the site of histone H1 attachment for a review on chro-
matin structure, see Kornberg (1977). The extremely low ADP-
ribosylation of the core histones (see sections 1b and 1c) indi-
cates that such modification has no role in maintaining the nuc-
leosome structure.

Smulson and his colleagues (Mullins et al., 1977; Giri et al.,
1978b) have shown that the poly(ADP-ribose) synthetase in
HeLa cell chromatin is located in the linker region and the extreme end of linker DNA. The enzyme activity was shown
not to coincide with the position of core particles on sucrose gra-
dients, but was present at a position enriched in mononucleo-
somes possessing linker regions (Giri et al., 1978b). Polyaclryl-
amide-gel analysis after separation of mono- and di-nucleo-
somes also showed that dimers and monomers containing linker regions possessed poly(ADP-ribose) synthetase activity (Giri et
al., 1978b).

The modification of nuclear proteins in nuclei and chromatin
prepared from mid-S-phase HeLa cells has been investigated by
Jump et al. (1979). Nuclease digestion of S-phase nuclei resulted
in the release of nucleosomes enriched in ADP-ribosylated pro-
teins and poly(ADP-ribose) synthetase activity as well as
nascent DNA from the DNA replicating fork. The results show
that the poly(ADP-ribose) synthetase activity is correlated with
extended forms of chromatin undergoing DNA replication or
repair (Jump et al., 1979).

Besides determining the probable localization of the poly(ADP-ribose) synthetase activity within chromatin, Smul-
on and co-workers (Mullins et al., 1977; Giri et al.,
1978a,b) have also looked at the proteins that are ADP-ribosylated both in vivo and in nuclei. When HeLa nuclei were incubated
with NAD+ before nuclease digestion and subsequent nucleosome analysis it was seen that ADP-ribosylated histones were prefer-
entially associated with mono- and di-nucleosomes, whereas higher oligonucleosomes exhibited more extensive modification
of non-histone proteins. The authors suggested that ADP-ribo-
sylation of histones either occurs in nucleosomes that are more
susceptible to nuclease or renders them more susceptible (Giri et
al., 1978a). Also, when the ADP-ribosylated proteins were analys-
ed in whole nuclei or in various classes of nucleosomes, it was
observed that in nuclei, histones H1 and H2B were the
major acceptors, with histones H2A, H3, HMG protein and M1
and M4 proteins being modified to a lesser extent. However,
with mono-, di- and tri-nucleosomes, very little histone modifi-
cation occurred, except on histones H1 and H3.1, whereas
ADP-ribosylation of HMG protein and M1 and M4 proteins
was greatly enhanced. Thus it seems that the core histones are
only modified when the chromatin is in a native conformation
(i.e., in nuclei), and these results emphasize the importance of
ADP-ribosylation in allowing the chromatin structure to
react or interact with and modify core histones (Giri et
al., 1978a).

This native or higher-ordered chromatin structure and its rela-
tionship to poly(ADP-ribosylation has been further investi-
gated in Smulson’s laboratory (Butt et al., 1978, 1979; Giri et
al., 1978a). The specific activity of the poly(ADP-ribose) syn-
thesase rises to a maximum with chromatin of 8–10 nucleo-
somes in length and as the complexity of the chromatin
increases with respect to nucleosomes, it was observed that
nucleosomes can be organized into solenoids with approx.
6–9 nucleosomes per helical turn of the solenoid. A unique structure
of this native or higher-ordered chromatin has been observed by
Byrne et al. (1978a). A transiently condensing or stabilizing fold of chromatin fibres
between adjacent nucleosomes. The enzyme activity was shown
to be synthesized in HeLa-cell nuclei (Stone et al., 1977; Stone
et al., 1978a), and for rat liver chromatin (Strat-
ing et al., 1978), both observations showing preferential clea-
vage of chromatin by micrococcal nuclease at periodicity of 8
and 16 nucleosomes. The higher specific activity of poly(ADP-
ribosyl)ation in the octanucleosomes has been partially explained by the observation that more than 90% of the total
incorporation in such structures occurred on protein C, which
has a mol wt of 125.000. Analysis of nucleosomes less than nine
units in length showed the same pattern as octanucleosomes with progressively less protein C modification (Butt et
al., 1979). The suggestion has been made that protein C may be the poly(ADP-
ribosyl)ation synthetase (Butt et al., 1979; Jump et al., 1979), and it
has been speculated that the enzyme might be bound to
chromatin at a periodicity of eight nucleosomes or in the mid-
region of a 16-nucleosome structure (Butt et al., 1979).

The poly(ADP-ribose) modification of chromatin proteins
may function by influencing higher-ordered chromatin structure for the synthesis of DNA during replication or repair (Jump
et al., 1979). In view of the importance of histone H1 in main-
taining higher-ordered chromatin structure (Finch & Klug, 1976;
Renz et al., 1977; Thoma & Koller, 1977; Worcel & Brambati, 1977), an interesting possibility is that the modifi-
ation of histone H1 may influence chromatin structure, in part,
by causing cross-linking of histone H1 molecules located on
non-adjacent linker regions within the chromatin. A dimer com-
oplex of histone H1 comprising two histone H1 molecules in
association with a single chain of poly(ADP-ribose) has been
shown to be synthesized in HeLa-cell nuclei (Stone et al.,
1977). Also, by using soluble chromatin preparations from HeLa-cell
nuclei, Kidwell and his colleagues (Byrne et al., 1978) have
shown a correlation between induced chromatin condensation
and the extent of histone H1–polymer complex synthesis. Con-
istent with this observation are the results of Perella & Lea
(1978, 1979), which show that, in rat liver nuclei polyanimes
cause an increase in histone H1 ADP-ribosylation (and possibly
histone H1–polymer dimer synthesis), which is accompanied by
a decrease in ADP-ribosylation of the core histones. The specu-
lation has been made that the formation of the histone–H1–
poly(ADP-ribose) dimer complex may provide a mechanism for
transiently condensing or stabilizing folds of chromatin fibres
(Stone et al., 1977; Lorimer et al., 1977; Byrne et al., 1978).

The transient nature of the process has been inferred from the
observation that the histone H1–polymer complex accumu-

1980
lulation is inversely related to the poly(ADP-ribose) glycohydrolase activity in the nuclear synthesizing system (Lorimer et al., 1977). If the histone H1–polymer complex does function within the chromatin via a cross-linking mechanism, then the histone H1 within the complex must have the same or similar affinity for DNA as within the chromatin system. In view of this, it is noteworthy that an ADP-ribosylation stems from the ambiguous results obtained otherwise. The large number of new analytical techniques to study the reaction between mono(ADP-ribose) and poly(ADP-ribose) is the same enzyme that elongates the chain. Similarly, the relationship between the reaction is inversely related to the poly(ADP-ribose) glycohydrolase activity in the chromatin via a cross-linking mechanism, then the histone H1–complex (Stone et al., 1978a). Also consistent with this hypothesis is the observation that there is an increased ADP-ribosylation in poly(ADP-ribose) synthetase obtained from permeabilized cells are more meaningful than those obtained from isolated nuclei. Perhaps the most promising approach is to determine ADP-ribosylation of specific fractions of proteins throughout a cellular activity. The choice of a model system presents a problem, since changes in ADP-ribosylation usually occur during changes in cell status. Thus it is difficult to ascribe definitively an effect on a particular biological process. In view of this, it is noteworthy that an involvement in DNA repair seems to be a most likely candidate; it is also one of the easier systems to study in vitro. The use of inhibitor studies has been neglected. The main problem is the lack of specificity of compounds such as nicotinamide and thymidine. It is certain that the introduction of 3-aminoo- and 3-methoxy-benzamide, which are specific inhibitors of poly(ADP-ribose) synthetase (Purnell & W. J. D. Whish, unpublished work), should provide a useful probe for investigation.

Thus, despite many problems, a large number of sensitive analytical techniques are now available, and their use should rapidly increase our understanding of the cellular function of ADP-ribosylation.

Note Added in Proof. It has recently been shown by three groups that a purified poly(ADP-ribose) synthetase will both initiate and elongate poly(ADP-ribose) chains on exogenously added histones (see Purnell et al., 1980).

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(3) Conclusions

Despite a great amount of research, ADP-ribosylation of nuclear proteins, as yet, poses an underdetermined problem. In section 2, no clear-cut conclusion emerges as to the involvement of ADP-ribosylation in any one particular cellular function, with the possible exception of DNA repair. For elucidation of the role or roles fulfilled by a postsynthetic modification of proteins, the identification of the proteins modified is of paramount importance. The system is clearly heterogeneous; protein species are known to be modified in vitro and in vivo for a small proportion of the total ADP-ribosylation observed. Although it is dangerous to draw on data obtained from studies in vitro, the results of Rickwood et al. (1977) suggest that over thirty protein species are modified. This may be either an overestimate (isolation of nuclei may expose new acceptor sites) or an underestimate (acceptors may be lost during isolation). The next step in the understanding of the role of ADP-ribosylation is the determination of the biological role played by the proteins that are ADP-ribosylated. The existence of two types of linkages between ADP-ribose and protein may indicate that at least two functions are fulfilled by ADP-ribosylation (as suggested by Bredehorst et al. (1979)). It is still unknown whether different enzymes are responsible for the formation of these bonds or indeed if the initial ADP-ribose molecule is attached by the same enzyme that elongates the chain. Similarly, the relationship between mono(ADP-ribose) and polyl(ADP-ribose) is unknown; is mono(ADP-ribo­sylated protein a precursor or a degradation product of poly(ADP-ribosylated) protein, or are the two completely separate? If ADP-ribosylation serves only to modify an amino acid in an enzyme's active site or protein's binding site, why is the system capable of producing poly(ADP-ribose)?

Clearly, some approaches used to attempt the definition of the function of ADP-ribosylation are more likely to succeed than others. The large number of new analytical techniques to study ADP-ribosylation stems from the ambiguous results obtained with older methods. Thus the activities of poly(ADP-ribose) synthetase obtained from permeabilized cells are more meaningful than those obtained from isolated nuclei. Perhaps the most promising approach is to determine ADP-ribosylation of specific fractions of proteins throughout a cellular activity. The choice of a model system presents a problem, since changes in ADP-ribosylation usually occur during change in cell status. Thus it is difficult to ascribe definitively an effect on a particular biological process. In view of this, it is noteworthy that an involvement in DNA repair seems to be a most likely candidate; it is also one of the easier systems to study in vitro. The use of inhibitor studies has been neglected. The main problem is the lack of specificity of compounds such as nicotinamide and thymidine. It is certain that the introduction of 3-aminoo- and 3-methoxy-benzamide, which are specific inhibitors of poly(ADP-ribose) synthetase (Purnell & W. J. D. Whish, unpublished work), should provide a useful probe for investigation.

Thus, despite many problems, a large number of sensitive analytical techniques are now available, and their use should rapidly increase our understanding of the cellular function of ADP-ribosylation.

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