Meeting Report

# POLY (ADP-RIBOSE)
## A report on the EMBO Workshop held in Hamburg, Germany, March 27–29th 1972

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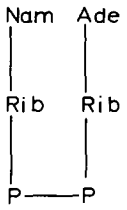
### 1. Introduction

Poly (ADP-ribose) (fig. 1) is an unusual new polymer found exclusively in the nuclei of nucleated cells. It was discovered in the laboratory of P. Chambon in Strasbourg. In 1963, Revel observed that the addition of nicotinamide mononucleotide (NMN) to renal nuclei *in vitro* stimulated the incorporation of adenine into acid-insoluble material. Chambon, Weill and Busch then demonstrated that the addition of NMN stimulated the incorporation of ATP in an RNA polymerase assay, even in the absence of the remaining three nucleotides. Their data suggested that NMN stimulated the synthesis of polyadenylic acid. In 1964 Janine Doly joined the group and she examined this system in some detail. This led to the isolation by Doly of a quite novel polymer: poly-(adenosine-diphosphoribose), (poly-ADP-ribose). The monomer sub-unit is ADP-ribose; and the units are bound by glycosidic bonds between the glycosidic carbon of the ribose to the 2′ hydroxyl of the ribose attached to the adenine. Most of the polymer is associated with chromosomal proteins, but about 10 to 20% is free polymer. The first surprise was that ATP is not the substrate for the synthesis of this polymer; the substrate is NAD. The enzyme breaks the NAD at the nicotinamide glycosidic bond and attaches the carbon 1′ of the ribose to carbon 2′ of the 'adenine' ribose in another molecule of NAD thus forming a polymer of ADP-ribose.

This unusual polymer has been known for only a

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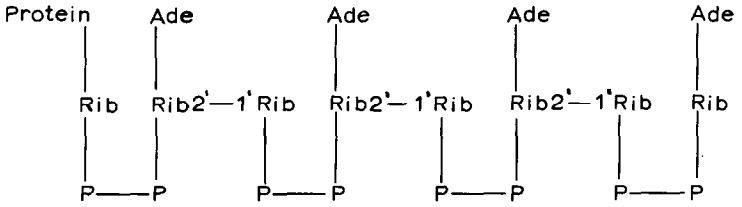


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relatively brief time, but it has excited a considerable burst of energy. The present consequences of this energy were reviewed in Hamburg on March 27 and 28, 1972 at the First International Workshop on the structure and function of poly (ADP-ribose). The workshop was organised by Professor H. Hilz of the University of Hamburg who has been amongst the most active participants in elucidating the properties of this new biopolymer. Thanks to generous support from EMBO, the Deutsche Gesellschaft für Biologische Chemie, Unilevers, E. Merck, Esso and B.P. it was possible to have representatives from every group active in the field.

## 2. Characterisation

The discovery and characterisation of poly (ADP-ribose) were elegantly described by Dr. J. Doly. She described also her attempts to demonstrate the existence of this polymer in intact cells. There appears to be only minute amounts of this polymer, and therefore it is not easy to provide unequivocal evidence of its existence in intact cells. However, she did demonstrate that there is most certainly an enzyme that can make this polymer in isolated nuclei. The enzyme is tightly bound to chromatin, and will use only NAD as substrate and is strongly inhibited by nicotinamide.

The existence of the polymer made with *in vitro* systems was powerfully demonstrated by Dr. M. Miwa (Tokyo) who has devised a large scale isolation of the polymer; from the nuclei isolated from 100 g of calf thymus he isolates 10 mg of completely pure poly-(ADP-ribose), starting with 100 mg of NAD. This development provides the possibility of a complete physical analysis of the structure of the polymer. Does this polymer show any secondary structure, such as base interactions or hydrogen bonding? The chain length of the polymer purified by Miwa was about 20 ADP-ribose units long.

K. Ueda (Kyoto) showed that the polymer appears to be attached to chromatin proteins; with about 70% to histones, mostly on histones F1 and F3. The polymer chains on these proteins are very short. The protein attachment is probably covalent because it is resistant to sodium dodecyl sulphate, to urea and to guanidinium hydrochloride; it is stable at pH 1 or 2 but is unstable in alkali. The half-time in 0.1 N NaOH at 0° is 5 min and at 37° it is completely hydrolysed in 1 min. However, the attachment to protein is heterogeneous; one set of polymer chains is labile to hydroxylamine, but a second set is only slowly cleaved. The two sets are about equal in amount. Only about 1 to 10% of histones contain ADP-ribose.

A nuclear-protein polymer may be isolated from an *in vitro* system (P. Adamietz, Hamburg). About 10 to 20% of the polymer radioactivity is soluble in 0.25 N hydrochloric acid. After several purification steps the complex is purified on DEAE-Sephadex in 6 M urea and 2 M NaCl, with a linear gradient of chloride from 0.2 to 0.5 M; the complex was bound more tightly than RNA or DNA and emerged at 0.41 M chloride. The complex may be separated on QAE-Sephadex into two fractions which elute at 0.37 and 0.41 M chloride, respectively. This polymer was more stable to alkali than that described by Ueda. It was not degraded at 37° in 1 hr in 0.25 N KOH. After 18 hr there was still 20% of polymer left. However, Ueda added at this time, that in his experiments the material that was not soluble in 0.25 N HCl was stable in alkali, in contrast to the lability of the acid-soluble material.

## 3. Biosynthesis and degradation

The requirements for DNA in a poly (ADP-ribose) polymerase *in vitro* system are striking. The poly (ADP-ribose) polymerase has an absolute requirement for DNA and histones in appropriate proportions. K. Yoshihara (Fukuoka) studied this dependence with an enzyme preparation purified 130 times from rat liver nuclei. This enzyme required a polynucleotide and histone. The relative activities with different nucleic acids were: rat liver DNA 600; poly A, poly U, poly C, poly G, poly dA, poly dG, 0; poly dG·dC, 130; poly dT · rA, 170; poly d(A–T), 260; poly dA + poly dT after heating and cooling, 2000. It seems that there is a requirement for a double stranded deoxyribose polymer containing A and T. This raises the question whether the poly (ADP-ribose) is involved in base pairing with DNA.

Since the poly-(ADP-ribose) chain has to be initiated as well as continued, the problem arises whether these two steps are catalyzed by a single enzyme. At the moment there is no evidence to answer this. However, we can ask whether the *in vitro* systems initiate

or merely elongate preformed small chains. K. Ueda demonstrated that DNAase treatment of an *in vitro* system decreased the incorporation of NAD into poly (ADP-ribose); the chain lengths decreased and this seemed to account for most of the decrease in incorporation. However, there are both more and longer chains formed at higher temperatures in the *in vitro* system (Hilz).

The degradation of poly-(ADP-ribose) has been elucidated both by M. Miwa working with T. Sugimura in Tokyo and by K. Ueda working with O. Hayaishi in Kyoto. Ueda described the degradation systems in rat liver. A phosphodiesterase hydrolyses the polymer to 2′-(5″phosphoribosyl)-5′AMP (PR-AMP) and AMP. The pH optimum is around 10 and the enzyme occurs in both cytoplasm and nucleus. It is inhibited by both GMP and AMP. In addition, rat liver contains a novel glycohydrolase which hydrolyses poly (ADP-ribose) to ADP-ribose. This enzyme has a pH optimum at pH 6.5–7.0, is inhibited by high ionic strength, and occurs in the nuclear sap and on chromatin. 4 mMolar 3′-5′-cyclic AMP inhibits the enzyme 50%; ADP-ribose also inhibits the enzyme. 0.5 M NaCl will release the enzyme from chromatin. Ueda summarised the work and views in the Kyoto laboratory by suggesting that DNA-histone (chromatin) may exist in an active, replicative form. In the presence of NAD, a hypothetical ADP-ribosylase will attach one monomer of ADP-ribose to histone and then poly (ADP-ribose) polymerase will elongate the chain. This process will be negatively controlled by the concentrations of nicotinamide, nicotinamide mononucleotide, thymidine and TMP. The resulting modified chromatin will be inactive for replication. The glycohydrolase is able to remove the polymer; this reaction, which is negatively controlled by cyclic AMP and ADP-ribose, regenerates the active DNA-histone again.

M. Miwa described the degradation processes as seen from the Tokyo laboratory. The rat liver phosphodiesterase is exonucleolytic from the AMP end of the polymer which contrasts with snake venom phosphodiesterase which is endonucleolytic. Miwa has found a glycohydrolase in calf thymus nuclei which is very similar to the rat liver nuclear enzyme. It gives oligo-(ADP-ribose) and ADP-ribose, but no PR-AMP. It is a glycohydrolase that works endo. It is mainly found in the nucleus and has been partially purified on phosphocellulose. This enzyme is mildly inhibited by high concentrations of cyclic AMP and more effectively by ADP-ribose.

## 4. Occurrence

Although poly (ADP-ribose) and its polymerase are studied mainly in rat liver, it does occur quite widely. Hilz (Hamburg) and Shall (Sussex) have studied it in various permanent cell lines in cell culture. Although it is not demonstrable in bacteria, it is present in the primitive, nucleated organism *Physarum polycephalum* (Shall). This organism, a myxomycete, related to the fungi is spontaneously synchronous in DNA synthesis and mitosis and very easy to grow. It is now widely used by molecular biologists, cell biologists and biochemists. The isolated nuclei of *Physarum* are able to synthesize acid-insoluble material from NAD: the reaction is DNA dependent and the properties of the system are very similar to those previously described in rat liver nuclei and to the enzyme in LS (mouse fibroblast) cells. The *Physarum* enzyme has an optimum temperature at 15° although the organism grows at 26°. The acid precipitable product that is formed in *Physarum* nuclei can be subdivided by extraction procedures: 40 to 60% of the acid precipitable product may be extracted with aqueous neutral buffer; of the remainder, about $\frac{1}{3}$ is extracted with 1 M $CaCl_2$ which extracts histones in this organism. The enzyme activity in the nuclei of mouse fibroblast (LS) cells is very similar in all its properties. The optimum temperature is 25° although the cells are grown at 37°. In this case, the unusual temperature optimum was clearly ascribed to a spontaneous instability of the system. Estimation of enzyme activity as a function of time enables an estimate to be made of the true initial rate as well as of the decay rate. Both the decay rate as well as the initial rate of enzyme activity are temperature dependent. Both reactions showed a simple temperature dependence, with linear Arrhenius plots. The decay is more sharply dependent on temperature than the initial rates of enzyme activity, and this explains the temperature optimum. Neither NAD or DNA protect the system from decay. The reaction kinetics yield a linear plot of the reciprocal of velocity against the reciprocal of substrate concentration. The estimated $K_m$ in mouse LS cells for NAD is 1.47±0.18 mmolar. The maximal velocity at 25° is $1.92\pm0.13 \times 10^{-15}$

moles of ADP-ribose incorporated/5 min/nucleus, which is equivalent to 128 nmoles of ADP-ribose incorporated/5 min/mg DNA. Nicotinamide inhibits the reaction with a $K_i$ of 14.3±1.0 μmolar.

## 5. Functions

The session on the function of poly (ADP-ribose) was introduced by a superb review of phosphorylation of nuclear proteins and the cell cycle by M.G. Ord (Oxford). In particular, she drew attention to the similarity of the problems presently facing those present at the meeting and the problems faced by those who elucidated the processes of phosphorylation of nuclear proteins. Ueda (Kyoto) offered the snippet that phosphorylation of histone leads to a 50% inhibition of *in vitro* poly (ADP-ribose) polymerase activity. This raises the possibility of an association between chromatin protein phosphorylation and the poly (ADP-ribose) synthesis. Perhaps the latter is yet another example of the covalent modification of chromatin proteins. Koide (Rockefeller, N.Y.) described their original observation which galvanised the people working with poly (ADP-ribose). Incubation of nuclei in the presence of NAD leads to the synthesis of poly (ADP-ribose). The ability of these pre-incubated nuclei to incorporate TTP into acid-insoluble material in a DNA polymerase assay was shown by L. Burzio and S. Koide to be depressed. This inhibition was dependent on the concentration of NAD in the pre-incubation step. They further adduced evidence that the DNA polymerizing enzyme was unaffected, but that the pre-incubation with NAD inactivated the template capacity of the chromatin. These observations were a focal point in the discussion of the possible physiological function of poly (ADP-ribose).

H. Hilz (Hamburg) in describing the extensive work from his laboratory offered an alternative hypothesis for the function of poly (ADP-ribose). He pointed out that most *in vitro* DNA polymerase assays do not correlate with *in vivo* incorporation of thymidine. Secondly, poly (ADP-ribose) activity in isolated nuclei is not correlated with *in vivo* increase in DNA or with thymidine incorporation. However, there is a clear correlation in his experiments between poly (ADP-ribose) polymerase activity and the *amount* of DNA. Indeed, if the polymerase activity is expressed per unit weight of DNA then it is remarkably constant, even when the total amount of DNA may vary 2-fold. Hilz concludes that poly (ADP-ribose) may participate in maintaining chromatin structure. These observations of Hilz emphasise a general problem; it is rarely demonstrated that *in vitro* DNA polymerase assays are indeed continuation of *in vivo* replication.

Hilz also showed that thymidine and some thymidine analogues inhibited the *in vitro* polymerase reaction. Thymidine, thymine, bromouracil, bromodeoxyuridine, dTMP and dTTP are all inhibitors. None of the three remaining bases or their derivatives were inhibitory. This inhibition may be of physiological significance. Thymine and nicotinamide are almost isosteric; the latter is a product of the reaction and a potent inhibitor. Hilz also reported that attempts to demonstrate the *in vivo* existence of poly (ADP-ribose) were inconclusive.

Two groups described the variation of poly (ADP-ribose) polymerase activity through the cell cycle. Smulson (Washington) synchronized Hela cells with a double thymidine (5 mM) block. Late S phase nuclei had a lower activity than mitosis or GI. Shall and colleagues (Sussex) examined the enzyme activity in isolated nuclei during the cell cycle in the spontaneously synchronous organism (*Physarum polycephalum*). There is a 2-fold decrease in enzyme activity per nucleus during the S phase, which follows mitosis directly in this organism. This observation requires further exploration, particularly in the light of the suggestion by Hilz which might predict that the enzyme activity would increase when DNA is made.

Both nicotinamide and 5-methylnicotinamide inhibit NAD glycohydrolase and poly (ADP-ribose) polymerase (J.B. Clark, London). Nicotinamide administration gives a very large increase in the total liver NAD content. 5-Methylnicotinamide does not have this effect. The increase in NAD content is therefore not due to inhibition of NAD degradation. In regenerating liver, there is an inverse relationship between NAD level and DNA content. After injecting nicotinamide (500 mg/kg body weight) there is a steady increase in NAD content and a corresponding decrease in DNA. No change is observed in RNA content, but the changes may be too small to be measured easily. Three hours after partial hepatectomy and after nicontinamide (500 mg/kg body weight), orotate incorporation was inhibited by about 70%. Twenty hours

after partial hepactectomy, orotate incorporation into both DNA and RNA was inhibited. Clark subscribed to a suggestion of Mandel made in 1959, that there is a competition for PRPP and ATP between RNA and DNA on one hand and NAD on the other. Koide retorted that ATP levels appear to be adequately maintained during DNA synthesis, so it was not clear why derivatives of ATP like PRPP should not also be adequately synthesized. In fact, in *Physarum* ATP levels increase during the period of DNA synthesis. The discussion at this point reflected a dichotomy between the view that one could explain the observations with simple metabolic interrelationships and the view that there were in addition specific regulatory processes which were the basis for the phenomena.

Haines (London) recalled earlier work where they demonstrated that in different classes of rat liver nuclei isolated by zonal centrifugation, the highest specific activity of NAD pyrophosphorylase occurred in those nuclei engaged in *in vivo* DNA synthesis, whereas the poly (ADP-ribose polymerase was most active in those nuclei in the G1 and G2 phases of the cell cycle. These results are exactly consistent with the observations in *Physarum*.

## 6. Metabolism of NAD

A final session reviewed the metabolism of NAD in relation both to poly (ADP-ribose) synthesis and to cell growth. Streffer (Freiburg) showed that the pyridine nucleotide synthetic cycle has a rapid turnover time, measured in minutes. The main inlet to the synthesis of NAD in rat and mouse is tryptophan. Several enzymes very early in this pathway are dramatically decreased in activity in tumour cells; and these enzymes start at low levels in new-born liver and rise during growth together with increase in NAD levels. It is not yet clear whether this is a tumour characteristic or if it is associated with changes in growth rate. The level of NAD in regenerating liver is low; if this level of NAD is increased by injecting nicotinamide then DNA synthesis is depressed.

Green (Sloan-Kettering, N.Y.) discussed the properties of NAD nucleosidase, which is an unusually interesting enzyme that is inactivated at pH 8.0 but not at pH 6.0. It is this cytoplasmic enzyme that is probably responsible for the rapid turnover of NAD. The activity of microsomal NAD nucleosidase is not affected by DNA. And in this connection Ueda (Kyoto) pointed out that diphtheria toxin does not produce polymers even in the presence of DNA. Diphtheria toxin is an enzyme that transfers ADP-ribose from NAD to ribosomal transferase II thus inhibiting protein synthesis. This unusual enzyme is clearly related to poly (ADP-ribose) polymerase. Günicke (Freiburg) discussed the regulation of NAD pyrophosphorylase, which synthesizes NAD or des-amido NAD from ATP and NMN or des-amido NMN, reversibly in fast growing cells but irreversibly in slow growing cells. The difference is due to the change in inorganic pyrophosphate. Thus, inorganic pyrophosphatase in nuclei may have a specially significant function. During nucleic acid synthesis there may be an accumulation of inorganic pyrophosphate which may reverse the synthesis of NAD and give rise to NMN and ATP. Further pyrophosphorylysis could give rise to nicotinamide and PRPP. Thus both ATP and PRPP could result.

Shall (Sussex) then described experiments which further strengthened the hypothesis that there is a correlation between growth rate and NAD content. The important aspect is the inverse correlation between NAD content and growth rate. This has previously been suggested in numerous animal experiments, and is present also in mouse fibroblast (LS) cells. An association between DNA replication and NAD pyrophosphorylase is observed in *Physarum polycephalum*. In the isolated nuclei of this organism the specific activity of this enzyme increases between 1 and $\frac{1}{2}$ hr before mitosis and reaches a peak at mitosis. The activity remains high throughout the S phase, which follows mitosis directly in this organism, and then declines to a stable basal level which is maintained throughout the G2 period. This fluctuation in activity is similar to that observed for DNA polymerase and thymidine kinase, but is unlike that of other metabolic enzymes in *Physarum*. During the S phase the ATP level rises steadily and there is no marked fluctuation in NAD levels through the cycle.

## 7. Physiological role

Theories of the physiological function of this new biopolymer were rampant because of the minimal experimental evidence that has accumulated till now.

The final session was devoted to a lively discussion of the contending opinions about the physiological role of poly (ADP-ribose). Koide advanced the possibility of an inverse relationship between poly (ADP-ribose) and DNA replication and was of the opinion that the polymer was related to DNA replication control. Hilz, on the other hand, felt that the good correlation between the poly (ADP-ribose) polymerase activity and DNA content suggested that the polymer participates in maintaining chromosome structure. Grünicke firmly drew attention to the possibility that poly (ADP-ribose) function may involve RNA synthesis. In support of this possibility I recall the work of Mandel and Revel that there is an inverse correlation between RNA synthesis and NAD levels. Shall suggested that NAD and poly (ADP-ribose) metabolism integrate cell growth, DNA replication and cell division.

Poly (ADP-ribose) has been discovered only recently. The field is in its infancy, and already it generates excitement and controversy. Major aspects remain to be elucidated. First and foremost, the physiological function of poly (ADP-ribose) and its polymerase must be established. Secondly, firm and unequivocal evidence of its existence *in vivo* is required. The physical and chemical properties of the natural polymer and the protein(s) to which it is bound in the nucleus require profound study. Does poly (ADP-ribose) play a part in transcription, replication, chromosomal structure or control of cell division? Whatever the outcome of these queries, the results will be exciting and illuminating to those interested in cell growth, cell replication and their control.

The workshop ended with a hearty and well deserved vote of thanks and appreciation to the sponsors of the meeting and to Professor Dr. H. Hilz and his colleagues for their initiative, impeccable arrangements and cordial hospitality.