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The poly ADP-ribose system in Triticum aestivus L.

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The Poly ADP-Ribose System in Triticum Aestivus L.

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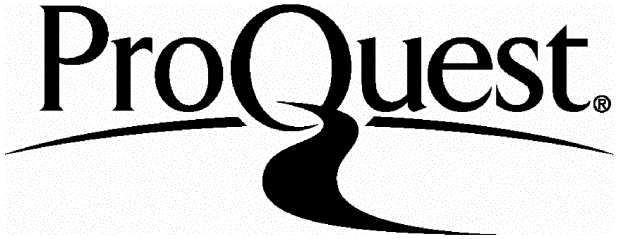
Andrew Whitby



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SUMMARY

It has been shown that nuclei isolated from wheat seeds can incorporate [^{3}H] NAD into an acid insoluble material which was subsequently shown to be poly (ADP-Ribose).

The poly (ADP-Ribose) polymerase responsible for this incorporation has been fully characterised, the enzyme having a pH optimum of pH 7.9 and an apparent temperature optimum of 17^{o}C. The enzyme requires free -SH groups for activity, with an optimal rate at a concentration of 10 mM β mercaptoethanol, magnesium also plays an important role in the enzyme activity, with the optimal Mg^{2+} concentration being 2 mM. The polymerase is 100% inhibited by 5 mM DTNB, and this inhibition cannot be reversed by the addition of β mercaptoethanol. Contrary to expectation the 100% inhibition of the polymerase by 0.5 mM Hg^{2+} is completely reversible upon addition of β mercaptoethanol. Two k_m were found for poly (ADP-Ribose) polymerase in wheat, 7.4×10^{-5} M and 5.7×10^{-6} M, and both nicotinamide and 3-amino benzamide were shown to be inhibitory, however the inhibition did not follow normal Michaelis-Menten kinetics. Activity was unaffected by the addition of DNA but was inhibited by AMP, cyclic AMP, ATP, GMP and GTP. The polymerase was shown to be unstable, the rate of decay following an Arrhenius relationship. The average chain length of poly (ADP-Ribose) formed by isolated wheat nuclei at an NAD concentration of 2.4 μM was 2.51 ± 0.09.

The isolated wheat nuclei were also shown to contain poly (ADP-Ribose) glycohydrolase activity, which was partially characterised. The degradation has pH optima at pH 6.4 and pH 8, and has an apparent temperature optimum of 34^{o}C. The enzyme activity was shown to be independent of added β mercaptoethanol and magnesium, however addition of DTNB caused some inhibition of the glycohydrolase.

It was found that the addition of the polyamines spermine, spermidine and putrescine to isolated wheat nuclei stimulated the formation of [^{3}H] poly (ADP-Ribose). Plant histones were extracted from the nuclear preparations and histones H1, H2A, H2B and possibly H3 were shown to be modified. The stimulation was shown to be independent of fluctuations in the poly (ADP-Ribose) glycohydrolase activity, changes in the average chain length of polymer synthesised, or increased specific labelling of any one histone. The increased ADP-Ribosylation was in fact due to a general increase in histone modification.

Finally the activities of poly (ADP-Ribose) polymerase and glycohydrolase were shown to vary during germination of isolated wheat embryos. The polymerase activity did not vary significantly during the time studied, however, over the same period of time (up to 12 hours) the glycohydrolase activity showed significant changes. After 6 hours of germination at least 96% of the glycohydrolase activity was lost, this loss being tentatively associated with the presence of an inhibitor of poly (ADP-Ribose) glycohydrolase produced *in vivo*.

ABBREVIATIONS

3AB	3 Amino benzamide
ADP	Adenosine diphosphate
ADP-Ribose (ADPR)	Adenosine diphosphate ribose
Ado	Adenosine
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
γBHC	gamma Benzene hexachloride
cyclic AMP (cAMP)	3', 5' cyclic adenosine monophosphate
DABA	1, 3 diamino benzoic acid dihydrochloride
DNA	Deoxribonucleic acid
DTNB	Dithionitrobenzoic acid
DTT	Dithiothreitol
GMP	Guanosine monophosphate
GTP	Guanosine triphosphate
NAD	Nicotinamide adenine dinucleotide
NMN	Nicotinamide mono nucleotide
Nm	Nicotinamide
PCA	Perchloric acid
PRAMP	2' - (5"-phosphoribosyl) - 5' - AMP
PPO	2, 5 - diphenyl oxazole
PTFE	Teflon
RNA	Ribonucleic acid
SDS	Sodium Dodecyl Sulphate
SVPDE	Snake Venom Phosphodiesterase
TCA	Trichloro acetic acid
TEA	Triethanolamine
Tris	2 - amino - 2 - hydroxymethyl propane 1, 3 - diol

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1.1. INTRODUCTION

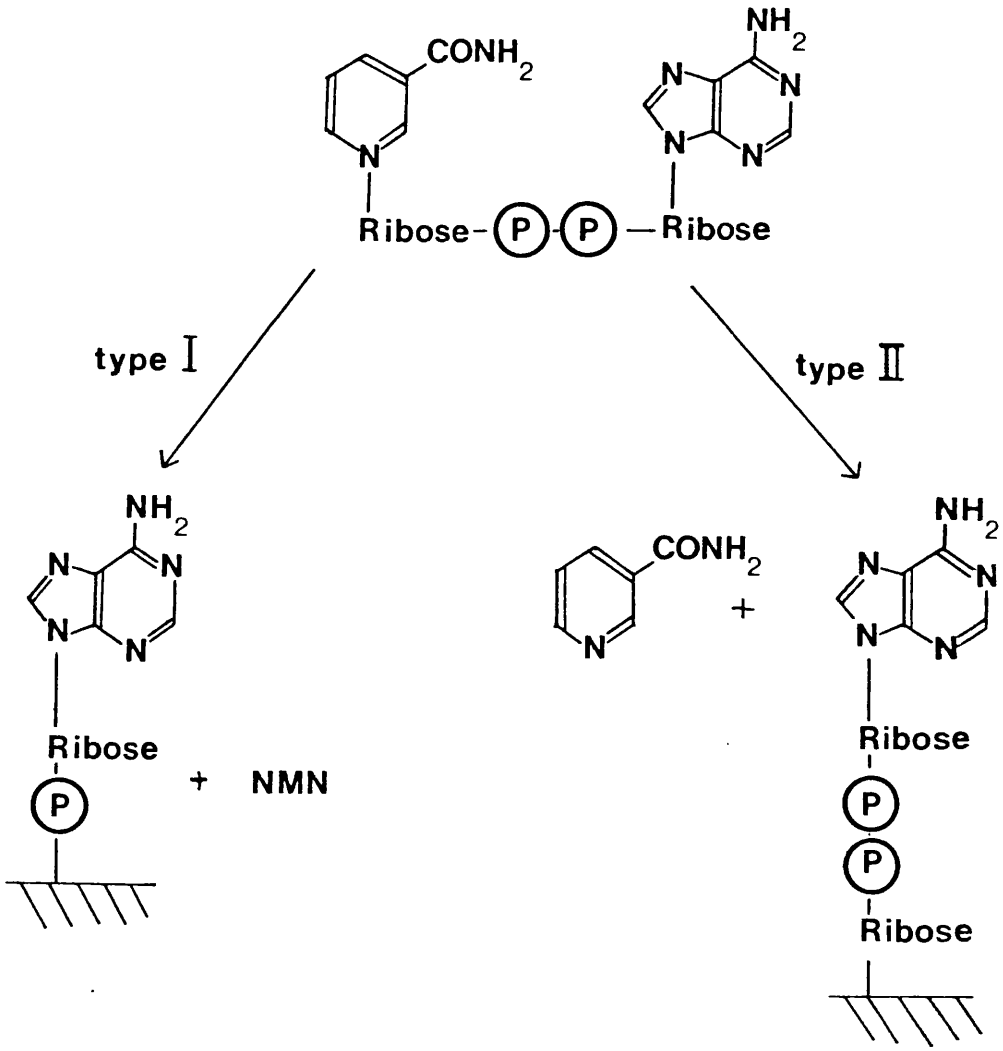
This introduction is intended to highlight recent advances in the field of poly (ADP-Ribose) metabolism and to give a brief outline of aspects of plant metabolism which are relevant to this study.

1.2. Poly (ADP-Ribose)

Biosynthesis of Nicotinamide Adenine Dinucleotide (NAD) in eukaryotes occurs in the nucleus, catalysed by the enzyme NAD pyrophosphorylase (E.C.2.7.7.1.) (Hogeboom and Schneider, 1952). The NAD molecule contains two energy rich bonds with a sufficiently high potential energy to permit group transfer reactions to occur. The two bonds are (i) a pyrophosphate bond, and (ii) a glycosidic link to the quaternary nitrogen atom of the pyridine ring of the nicotinamide moiety (Zatman et al, 1953). The two possible transfer reactions are referred to as Type I and Type II. (Fig. 1.1.). Transfer reaction Type I is typified by the reaction of NAD with polynucleotide ligase in Escherichia coli, which involves transfer of adenosine 5' monophosphate (AMP) to the enzyme, coupled with the release of nicotinamide mononucleotide (NMN) (Little et al, 1967), Olivera and Lehman, 1967). Transfer reaction Type II involves the transfer of the adenosine (diphosphate ribose) (ADP-Ribose) moiety to a suitable acceptor, coupled with the release of nicotinamide and a proton. Examples of this type of reaction are the exchange between nicotinamide analogues and nicotinamide, giving the corresponding NAD analogues (Zatman et al, 1953). Another such is the transfer of the ADP-Ribose moiety to the elongation factor EF-2,

Fig. 1.1. Transfer Reactions of NAD

(hatched area represents one or more acceptor proteins)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NH2
N
N
N
N
Ribose-P-P-Ribose
type I
type II
NH2
N
N
N
N
Ribose
P
+ NMN
CONH2
+
NH2
N
N
N
N
Ribose
P
P
Ribose

catalysed by Diptheria toxin, (Honjo and Hayaishi, 1973).

The transfer of ADP-Ribose to various receptor proteins, with a detailed discussion of the details and possible functions have been reviewed recently (Hilz and Stone, 1976, Hayaishi and Ueda, 1977, Shall et al, 1977). The information below gives only a brief outline and attempts to bring the information more up to date. For a detailed current review see Purnell et al (1980).

The formation of poly (ADP-Ribose), formed from repeating units of ADP-Ribose via glycosidic links (see below) as a product of incubating [^{3}H] ATP and NMN with a crude rat liver homogenate, was firstshown independently, and at approximately the same time by three separate laboratories (Chambon et al 1966, Nishizuka et al 1967, Sugimura et al 1967). It was then shown that NAD was the true substrate of the nuclear enzyme, and that the product was susceptible to cleavage by Snake Venom Phosphodiesterase (SVPDE) (E.C.3.1.4.1.), to a nucleotide containing two ribose and two phosphate residues per adenine moiety (Chambon et al, 1966, Nishizuka et al, 1967, Reeder et al, 1967, Hasagawa et al, 1967).

The structure and conformation of the polymer have been investigated using a combination of physical and chemical techniques, notably NMR spectral analysis and specific enzyme digestion. The initial chemical analyses were performed by Doly and Petek (1966), Chambon et al (1966), Nishizuka et al (1967) and Reeder et al (1967). These indicated that only one of the two riboses in the ADP-Ribose moiety had free adjacent hydroxyl groups at positions 2' and 3', phosphatase treatment yielded ribosyl-adenosine, and methylation followed by acid hydrolysis demonstrated a 1' - 2" glycosidic link. Analyses

of the polymer (Miwa et al, 1977) and the monomer (Ferro and Oppenheimer, 1978) by NMR have shown the polymer to be formed of repeating units of (1" - 2') - α - D - ribofuranosyl-adenosine-5', 5" - bis (phosphate) (Fig: 1.2.) Suhadolnik et al (1977) have also shown that a 1" - 3' osidic linkage is possible by the use of the NAD analogue, 2-deoxy NAD. Separation and analysis of long chains of poly (ADP-Ribose) (Tanaka et al, 1978) led to the suspicion that a branched form of the polymer existed, and this has recently been confirmed, by Miwa et al (1979) who proposed a branch approximately once per 20 - 30 residues, and identified the branch linkage as 2' - 1" - ribosyl - 2" - (or 3" -) (1 - ribosyl) adenosine - 5', 5", 5 - tri (phosphate). Inagaki et al (1978) have also studied the conformation of the polymer in solution using NMR.

The occurrence of poly (ADP-Ribose) has been widely reported in several animal tissues e.g. rat (Nishuzuki et al, 1967, Burzio and Koide, 1972), pigeon erythrocytes (Nishizuka et al, 1967), mouse L cells (Hilz and Kittler, 1971, Shall et al, 1972, Lehman and Shall, 1972), pig (Lehman et al, 1974, Janaki-devi and Koh, 1974), human tissue (Preiss et al, 1971, Burzio et al, 1975), cattle (Okayama et al, 1977, Ito et al, 1979,) birds (Chambon et al, 1966, Muller et al, 1974), fish (Sugimura 1973) and Physarum polycephalum (Brightwell and Shall, 1971). Various bacteria have been examined (Nishizuka et al, 1967) without any success in identifying the polymer or the enzymes concerned with its metabolism (see below). Soon after the commencement of this study there were two tentative identifications of poly (ADP-Ribose) in plants. Payne and Bal (1976) reported a fixation of [^{3}H] NAD by the nuclei of onion cells,

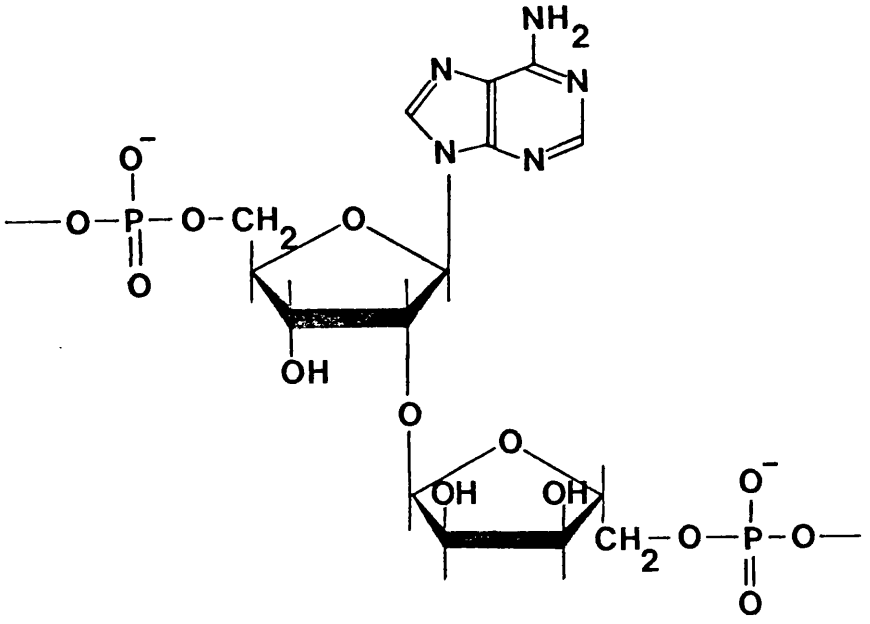
Fig. 1.2.A. Nature of the glycosidic linkage in poly (ADP-Ribose

Fig. 1.2.B. Scematic Representation of poly (ADP-Ribose) and the site of action of enzymes associated with poly (ADP-Ribose) metabolism

Key:-

1. Initiating enzyme?
2. Poly (ADP-Ribose) Polymerase.
3. Phosphodiesterase.
4. Poly (ADP-Ribose) Glycohydrolase.
5. ADP-Ribose Protein Hydrolase?

A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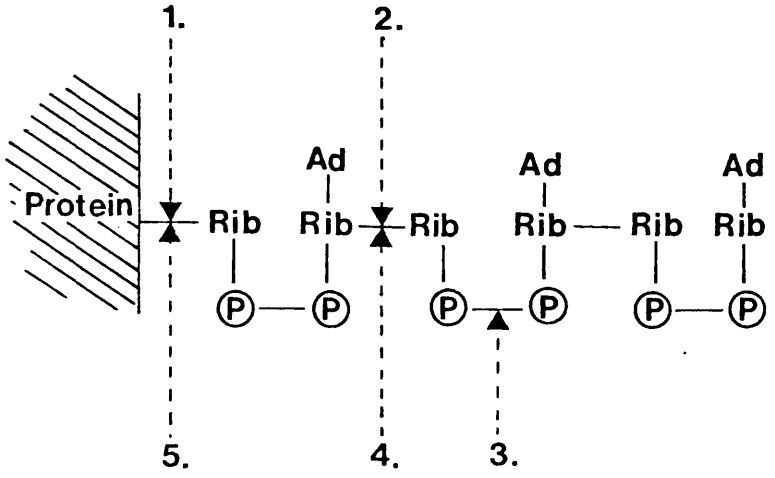
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using autoradiographic methods, and Lin (1976) reported a poly (ADP-Ribose) - like synthesising ability in rice. Although Lin did not fully characterise this ability, the conditions given (e.g. effect of Mg^{2+}, and nicotinamide) indicate that it may have been due to poly (ADP) Ribose formation.

The synthesis and degradation of poly (ADP-Ribose) may involve four or more enzymes (Fig. 1.3.). The general reaction catalysed is:-

$$n\ NAD^{+} \longrightarrow \left(\text{ADP-Ribose}\right) n + nNm + nH^{+}$$

The enzyme activity which catalyses the transfer of ADP-Ribose moieties is an ADP-ribosyl transferase, and has been termed poly (ADP-Ribose) polymerase, poly (ADP-Ribose) synthetase and poly (ADP-Ribose) synthase. It is at present unclear whether the enzyme catalyses the first step of ADP-Ribosylation, i.e. attaching the initial ADP-Ribose residue to an acceptor protein, or just acts as an "Elongase", and a separate enzyme, an "Initiase", then performs the first step. This latter view is supported by the work of Ueda et al (1979) who used a Histone H1 - poly (ADP-Ribose) adduct (Kun et al, 1976), showing that the adduct was primarily elongated, and only a small degree of initiation was found.

The observations of Kidwell and Burdette (1974) show that nuclei from S phase vs G2 phase HeLa cells have differing Km values for NAD, and that the Km for the formation of short poly (ADP-Ribose) residues is different to that for longer polymer chains, (Dietrich and Siebert, 1973). Observations from the laboratory of Hilz showing two different ADP-Ribose acceptor linkages (Adamietz and Hilz, 1976, Adameitz et al, 1978, Wielkins et al, 1979) also tend to indicate more than one enzyme is involved.

There are two enzymes implicated in the degradation of poly (ADP-Ribose). The first is an exoglycosidase, splitting 1' - 2" ribose bonds, called poly (ADP-Ribose) glycohydrolase (Miwa and Sugimura, 1971, Ueda et al, 1972a, Tanaka et al, 1976). The other enzyme is a phosphodiesterase, acting on the pyrophosphate bond, releasing 2' - (5" - phosphoribosyl) - 5' - AMP (PRAMP), Futai et al, 1967).

The properties of poly (ADP-Ribose) polymerase have been examined in detail in many cases, e.g. rat liver (Fujimura et al, 1967), LS cell nuclei (Shall et al, 1972), Physarum Polycephalum (Brightwell and Shall, 1971), Baby Hamster Kidney (BHK) cells (Furneaux and Pearson, 1977) and rat pancreas (Poirer et al, 1973).

The pH optimum is around pH8 in all systems examined, Mg^{2+} ions are needed for maximal activity, although the optimum Mg^{2+} concentration is dependent on the NAD concentration. Reduced -SH groups are also required for optimal activity. The poly (ADP-Ribose) polymerase seems to exhibit a high substrate specificity, and appears to be reversibly inhibited by nicotinamide. The affinity of the enzyme for its substrate (NAD) differs between tissues, and possibly between cells taken at different stages of the cell cycle, or at different degrees of purification of the enzyme. Values have been obtained in a range of $2\text{-}4 \times 10^{-4}$ M from Ehrlich Ascites cells (Romer et al, 1968) to 1.5×10^{-3} M from LS cells (Stone and Shall, 1973). When in a purified state (see below) the polymerase needs added DNA for maximal activity, although from the results obtained from nuclear preparations it is not at all clear whether this is so. (For complete discussion see Hilz and Stone 1976.)

The degradation of poly (ADP-Ribose) *in vivo* is thought to occur via the glycohydrolase, as the optimum pH for the phosphodiesterase has a very high, sharp pH optimum of pH 10, significantly above physiological conditions. The degradation of the poly (ADP-Ribose) *in vitro* by snake venom phosphodiesterase (SVPDE) has been used extensively to characterise poly (ADP-Ribose) (see below).

The degradative enzyme, poly (ADP-Ribose) glycohydrolase, has been partially purified from calf thymus by Miwa and Sugimura (1972), rat liver (Ueda *et al*, 1972a, 1972b) and Physarum polycephalum by Tanaka *et al* (1976). The pH optimum is about pH 7.5 in calf thymus and rat liver, and pH 6 in Physarum. The enzyme is inhibited by a range of nucleotides:- ADP, ATP, ADP-Ribose, 3', 5' cyclic AMP, as well as $(NH_4)_2\ SO_4$. The enzyme prepared from calf thymus was also inhibited by calf thymus histones, H2A, H2B and H3, the inhibition with H2A being reversed by DNA (Miwa *et al*, 1974).

It is apparent that the extent (and possibly the direction) of poly (ADP-Ribose) synthesis is governed by the relative synthesis and degradation activities, and it is important to consider both when attempting to determine the extent of poly (ADP-Ribosylation).

The detection and quantitation of poly (ADP-Ribose) *in vitro* has been greatly facilitated by the use of snake venom phosphodiesterase. The average chain length of polymer formed can be estimated from the amount of radioactivity found in AMP, compared to that in PRAMP and AMP after SVPDE digestion. As the AMP residues arise only from the terminal ADP-Ribose residue, the average chain length is given by:-

$$\text{Average chain length} = \frac{(\text{cpm in AMP}) + (\text{cpm in PRAMP})}{(\text{cpm in AMP})}$$

(Nishizuka et al, 1969)

This determination must be carried out using SVPDE free of phosphomonoesterase activity. It must also be noted that Farzaneh and Pearson (1978) have also shown that in vitro elongation of in vivo synthesised chains occurs, which will lead to low values for average chain lengths. The average chain lengths obtained will also be influenced by any endogenous enzymes, e.g. poly (ADP-Ribose) glycohydrolase, phosphodiesterases, phosphatases, present during the incubation.

Poly (ADP-Ribose) has been quantified by several other methods. By isotope dilution methods from the laboratory of Hilz (Hilz et al, 1972); electrophoretic techniques have been used to estimate average chain length, coupled with $CsSO_4$ - guanidinium/urea density gradient centrifugation, by Hilz and co-workers (Adamietz and Hilz, 1976, Adamietz et al 1978b), and electrophoresis has also been used in Sugimura's laboratory (Tanaka et al, 1978) for chain length estimations, leading to the discovery of 'branched' poly (ADP-Ribose) (see above). Recently fluorimetry has been applied to the quantitation of poly (ADP-Ribose) by Niedergang et al (1978), via the conversion of polymer to mono (ADP-Ribose). However, the most significant recent advance has been the development of immuno-chemical assays for poly (ADP-Ribose) and mono (ADP-Ribose). The approach adopted by Hilz and co-workers has been to convert poly (ADP-Ribose) to mono (ADP-Ribose), and then to convert this to 5' AMP by mild base treatment. The 5' AMP is then assayed using a highly specific antibody. (Bredehorst et al, 1978a, 1978b). Ferro et al (1978) have prepared antibody to poly (ADP-

Ribose) with an average chain length of 4 or greater. Sugimura and his co-workers have prepared and characterised antibodies to PRAMP i.e. mono (ADP-Ribose), and poly (ADP-Ribose) (Sakura et al, 1978), Kanai et al, 1978a, 1978b).

The majority of studies in the field have dealt with in vitro situations, but recently in the laboratories of Berger and Shall, techniques involving the use of nucleotide permeable cells have been developed in an attempt to approach a true in vivo situation. These studies have shown poly (ADP-Ribose) polymerase activity (Berger et al, 1978a, 1978b, 1978c, 1978d, 1979, Halldorsson et al, 1978), and the technique has also been used to follow the polymerase activity during the cell cycle (see below).

The nature of the acceptor, or acceptors, of poly (ADP-Ribose) has received much attention. Early studies (Hilz and Stone, 1976) indicated that a high percentage of acceptors were histones, however non-histone proteins are also modified.

Hilz and co-workers have shown that at least two types of modification occur, and these can be distinguished by the effect of neutral NH_2OH, one linkage being susceptible to hydrolysis and one resistant, (Bredehorst et al, 1978c, Wielckins et al, 1979). Bredehorst et al have also shown that the bulk of modification in vitro is mono (ADP-Ribose), and when modifications in vitro are compared with those in vivo, using HeLa cells and immunochemical techniques (see above), they have shown that in vivo modification of histone H1 is mainly poly (ADP-Ribose), whilst in vitro it is mono (ADP-Ribose) (Adamietz et al, 1978b).

The sites of modification have been extensively studied using several organisms. Hayaishi and co-workers, using rat

liver nuclei, have shown that Non-Histone Proteins (NHP), Histones H2B and H1 are modified (Okayama et al, 1978) and also protein A24 (histone 2A + ubiquinone) (Hiroto and Hayaishi, 1978). Ord and Stocken (1977) have shown that in rat liver nuclei histones H1, H2A, especially H2B and very little H3 are modified. Using trout testis Poirer has shown that histones H1 and H6 (trout specific histone) are modified, but no other histones (Wong et al, 1977). The same group have also examined nuclei from rat pancreas, and the only histone found to be modified was H1. Workers in the laboratory of Smulson have examined the modification of HeLa nuclei, and have shown that the major acceptors are histones H1, H2A, H2B and H3. High mobility group (HMG) proteins and nuclear proteins M1, M2, M3 and M4 are slightly modified, but no modified histone H4 was found. The same group have also shown that the pattern of distribution is dependent upon the chromatin being in its native conformation. When nuclei are taken from only S phase cells a very similar pattern is found (Giri et al, 1978, Jump et al, 1979). Riquelme et al, (1979a, 1979b) have found a very simlar pattern of modification using purified rat liver nuclei. Using various enzymatic and chemical steps they have shown that the glutamic acid residues numbers 2 and 116 of the polypeptide chain of histone H1 are the probable modification sites, and they also propose that there is a mono (ADP-Ribose) on the -COOH of glutamic acid residue number 2 of histone H2B. Histone has also been found to form a complex with long chains of poly (ADP-Ribose), forming a "dimer" of 2 H1 with poly (ADP-Ribose) with an average chain length of 15 (i.e. 7-8 units of ADP-Ribose per molecule of H1). So far this dimer has only

been shown to exist in vitro (Stone et al, 1977).

The biological role of poly (ADP-Ribose) is still unknown, the accumulated evidence tends to indicate a close involvement with chromatin activities. It is also becoming apparent that there is more than one in vivo role for poly (ADP-Ribose) as proposed by both Hilz and Hayaishi (Wielkins et al, 1979, Hayaishi et al, 1979).

There have been several studies following the cell cycle and attempting to correlate DNA and poly (ADP-Ribose) synthesis. Berger et al have used permeabilised systems to investigate this, using mouse L cells (Berger et al, 1978a, 1978b), hamster ovary cells (Berger et al, 1978c) and human lymphocytes, both normal and chronic leukaemic (Berger et al, 1978d). In each case they have found a good correlation. DNA synthesis is at a maximum in S phase, being 4-5 fold greater than during G1 and G2, whilst poly (ADP-Ribose) synthesis is the opposite, being at a minimum during S phase. The chronic leukaemic lymphocytes (CLL) were the only case not to show a correlation, Poly (ADP-Ribose) synthesis is dissociated from DNA synthesis in CLL cells. These observations (except for CLL) are confirmed by Tanaka et al (1978). Using an in vitro assay system for HeLa cells, this study also showed that the apparent net synthesis of poly (ADP-Ribose) is not due to a change in the degree of polymer degradation. Several workers have shown poly (ADP-Ribose) to be implicated in inhibition of template activation (Burzio and Koide, 1971, 1973, Yoshihara and Koide, 1973a). Release of DNA polymerase from nuclei has also been observed (Yoshihara and Koide, 1973b), as has inhibition of DNA polymerase (Nagao et al, 1972) and the modification and inhibition of a Mg^{2+} dependent

endonuclease (Yoshihara et al, 1974, Yamada et al, 1975). Recently Tanigawa et al (1978a, 1978b) have suggested that DNA synthesis is stimulated by poly (ADP-Ribose) formation, caused by an increase in accessibility of the DNA to the DNAse, resulting in template activation.

The involvement of poly (ADP-Ribose) in DNA repair mechanisms has recently been independently proposed by three groups of workers in the laboratories of Berger, Shall and Smulson. Shall and co-workers have shown that if mouse L cells are exposed to streptozotocin (an alkylating agent), 5-methyl nicotinamide and theophylline, a significant drop in the amount of NAD per cell is observed, with a corresponding increase in poly (ADP-Ribose) formation (Davies et al, 1977a). They have also shown that if non-cytotoxic inhibitors of poly (ADP-Ribose) polymerase are administered with the streptozotocin there is no drop in the NAD level. (Davies et al, 1976). The same group of workers have shown a similar effect with L1210 cells, using γ-radiation to cause the drop in NAD levels, breaks in the DNA were demonstrated by alkaline-sucrose gradients. γ -radiation is thought to cause single and double strand cleavage of DNA (Beerman and Goldberg, 1974). Shall and co-workers suggest that DNA damage causes flux through poly (ADP-Ribose), probably related to DNA repair (Davies et al, 1977b). Smulson and co-workers have used HeLa cells for a similar study. They used the alkylating agents N-methyl-N-nitrosourea(NMU) and 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU). NMU is also a recognised anti cancer agent. Like Shall et al, Smulson found that exposure to alkylating agents caused a drop in NAD levels, accompanied by a stimulation of poly (ADP-Ribose) polymerase. A stimulation of the poly-

merase was also observed when isolated nuclei were treated with DNAse I, but if the nuclei were pretreated with NMU before DNAse I no stimulation was observed unless a recovery period was allowed after NMU treatment (Smulson et al, 1977). More recently this group have shown that this increase in poly (ADP-Ribose) formation is not due to a change in poly (ADP-Ribose) glycohydrolase activity, or chain elongation, indicating that NMU increases the accessibility of the nucleosome core histones to poly (ADP-Ribose) modification. They have also postulated the CCNU acts in the main by modifying non-histone proteins (Sudhakur et al, 1979a, 1979b). Berger et al (1979) have shown, using permeable normal human lymphocytes and X-radiation to cause DNA damage, that the increase in both poly (ADP-Ribose) synthesis and DNA synthesis is cycloheximide insensitive.

Another aspect of metabolism to which poly (ADP-Ribose) has been linked is differentiation. The proposals by Morton (1958, 1961) that the intracellular concentration of NAD may be a controlling factor in cell division (NAD levels are low in rapidly dividing tissue, high in resting tissue) and the work of Rechsteiner et al (1976) indicating that about 95% of NAD degradation which occurs in a cell occurs in the nucleus, presumably via poly (ADP-Ribose) has aroused significant interest. Caplan has shown that poly (ADP-Ribose) synthesis is closely related to differentiation of chick mesodermal cells into muscle or cartilage (Caplan and Rosenberg, 1975, Caplan, 1976). More recently, reports by Claycomb (1976a, 1976b), using cardiac muscle tissue from neonatal rats show that intracellular NAD concentrations are inversely related to DNA synthesis. Further indirect evidence has come from the work of Ghani and Hollenberg (1978a,

1978b) again using cardiac tissue. They found that if neonatal cardiac tissue is incubated in oxygen enriched conditions (20% compared to 5%) the rate of cell division is markedly reduced. It was also observed that increasing the oxygen level stimulated poly (ADP-Ribose) synthesis (1978a). Ghani and Hollenberg then proposed that the control of the rate of cell division by oxygen acts by altering the redox state of a metabolite and they suggested that $NAD^+ \rightleftharpoons$ NADH was being altered, thus controlling the availability of substrate for poly (ADP-Ribose) polymerase. Ghani and Hollenberg also noted that the kinetic parameters (Km, Vm) for poly (ADP-Ribose) polymerase were significantly different when cells were grown in 5% oxygen or 20% oxygen. The values obtained were, in 5% oxygen, Km of 0.5 mM, Vm of 25 cpm, and in 20% oxygen, Km of 0.043 mM, Vm of 250 cpm. They proposed that poly (ADP-Ribose) metabolism does participate in the regulation of heart cell division, but probably by several different mechanisms.

A more recent report (Morioka et al, 1979) has suggested that the level of poly (ADP-Ribose) synthesis is regulated by some factor (or factors) secreted by Friend Leukaemia (FLC) in culture medium, i.e. the level of poly (ADP-Ribose) synthesis is correlated with differentiation of FLC. A similar observation has also been reported by Yamada et al (1978) using mouse L cells, where a correlation between poly (ADP-Ribose) syntheis and differentiation was found.

Porteous et al (1979) have utilised an intestinal cell system to examine the role of poly (ADP-Ribose) during cell division. The system used can be taken to be comprised of two distinct cell types, regenerating (and dividing) cells of the

lower crypt, and differentiating (but not dividing) cells of the upper crypt. Both cell types were shown to possess the *in vitro* capability of forming poly (ADP-Ribose), although the majority of enzyme activity was located in the dividing lower crypt cells. The same report also deals with the location and type of modifications occurring (i.e. poly or mono ADP-Ribosylations), differences being apparent between the two cells types. This study tends to support the views of other works, outlined above, and may lead to an insight into how NAD levels affect dividing cells.

One other aspect of metabolism which has lately aroused interest is the question of whether poly (ADP-Ribose) modifies any other nuclear enzymes. Furneaux and Pearson (1978) have shown, using nuclei from baby hamster kidney cells, that poly (ADP-Ribosylation) of nuclear proteins inhibits RNA polymerase 1, but stimulates RNA polymerase II. Also Leone *et al* (1979) have recently reported that mammalian testis poly (ADP-Ribose) polymerase is stimulated by seminal and pancreatic RNAses, with a parallel inhibition of the RNAse activity.

In addition Suzuki and Murachi (1978) have found that either poly (ADP-Ribose), or a similar compound, inhibits a chromatin associated neutral protease. Attention has also been paid recently to the effect of the chromatin structure on poly (ADP-Ribose). For details of the early studies which showed the poly (ADP-Ribose) polymerase to be located in the nucleus see Hilz and Stone (1976) and Hayaishi and Ueda (1977). Recent reports have shown that the pattern of modification of nuclear proteins is dependent upon the conformation of the chromatin (Giri *et al*, 1978a), which is supported by the work of Byrne

et al (1978), who related the effect of polyamines in vitro to changes in chromatin condensation. Smulson and colleagues have shown that poly (ADP-Ribose) polymerase activity is primarily associated with template active regions of chromatin (Mullins et al, 1977) and that there is a relationship between chromatin organisation and poly (ADP-Ribose) polymerase activity (Butt et al, 1978). Lately they have also shown that the poly (ADP-Ribose) polymerase is located in the internucleosome linker region (Giri et al, 1978) and that a nucleosome octomer is necessary for optimal polymerase activity (Butt et al, 1979).

In addition to the above, there are also several non-nuclear systems involving ADP-Ribose, including mitochondrial and cytoplasmic systems, Diptheria toxin elongation factor EF2, and modification of the RNA polymerase of T_4 infected E. coli, all of which are well covered by both Hilz and Stone (1976) and Hayaishi and Ueda (1977). When the sera of patients suffering from Systemic Lupus Erythrocytes (S.L.E.) is examined it has been found to contain significant titres of antibody to poly (ADP-Ribose) (Kanai et al, 1977, Okolie and Shall, 1979) although the clinical significance is, as yet, unknown.

1.3. Nucleotide Metabolism in Plants

Although purine metabolism in higher plants has received some attention, and has recently been reviewed (Suzuki and Takahashi, 1977) relatively few recent reports concerning pyrimidine metabolism in higher plants are to be found, although several reports have shown the presence in plants of all the pyrimidine nucleotides and nucleosides found in animal tissue. (Brown and Silver, 1966, Taniguchi et al, 1967, Brinkman et al.

1972, Collins et al, 1972a, 1972b, Yuan, 1976).

The metabolism of the pyrimidine nucleotides and nucleosides has been studied in detail, in pear, by Brown and Mangat (1967), Silver and Gilmore (1968), Rose et al (1968) and Ashihara (1977), in germinating pine pollen by Nygaard (1973) and in wheat by Grzelczak and Buchowicz (1975). The metabolism of NAD and its derivatives has been studied in several higher plants (see below).

Several studies have shown that quinolinic acid (2, 3, pyridine-dicarboxylic acid) is a common precursor to many of the pyridine ring compounds in plants (Hadwiger et al, 1963), animals (Gholson et al, 1964) and micro-organisms (Andreoli et al, 1963). Amongst these compounds derived from quinolinic acid are NAD and other intermediates of the Preiss-Handler cycle (see fig. 1.3.) (Preiss and Handler, 1958a, 1958b) and the alkaloid ricinine (Waller and Yang, 1965, Waller et al, 1966, Hiles, 1969). The studies on ricinine have reinforced the hypothesis that NAD synthesis and degradation are via the cyclic Preiss-Handler pathway in plants. The metabolism of NAD has been studied in detail in normal wheat leaves (Godavari and Waygood, 1970), in bean leaves (Chen et al, 1974) and in Puccina infected wheat leaves (Pao et al, 1974). More recently Barz and co-workers have reported, in a series of papers, on the metabolism of nicotinic acid, nicotinamide and their derivatives in continuous plant cell cultures (Leienbach and Barz, 1976, Heeger et al, 1976, Leienbach et al, 1976, Neuhann et al, 1979). Their work has shown that NAD metabolism is via the Preiss-Handler cycle, but that one of two derivatives of nicotinic acid (N methyl nicotinamide and N-α-L-arabino-nicotinamide acts as a reservoir for nicotinic acid (and therefore NAD)

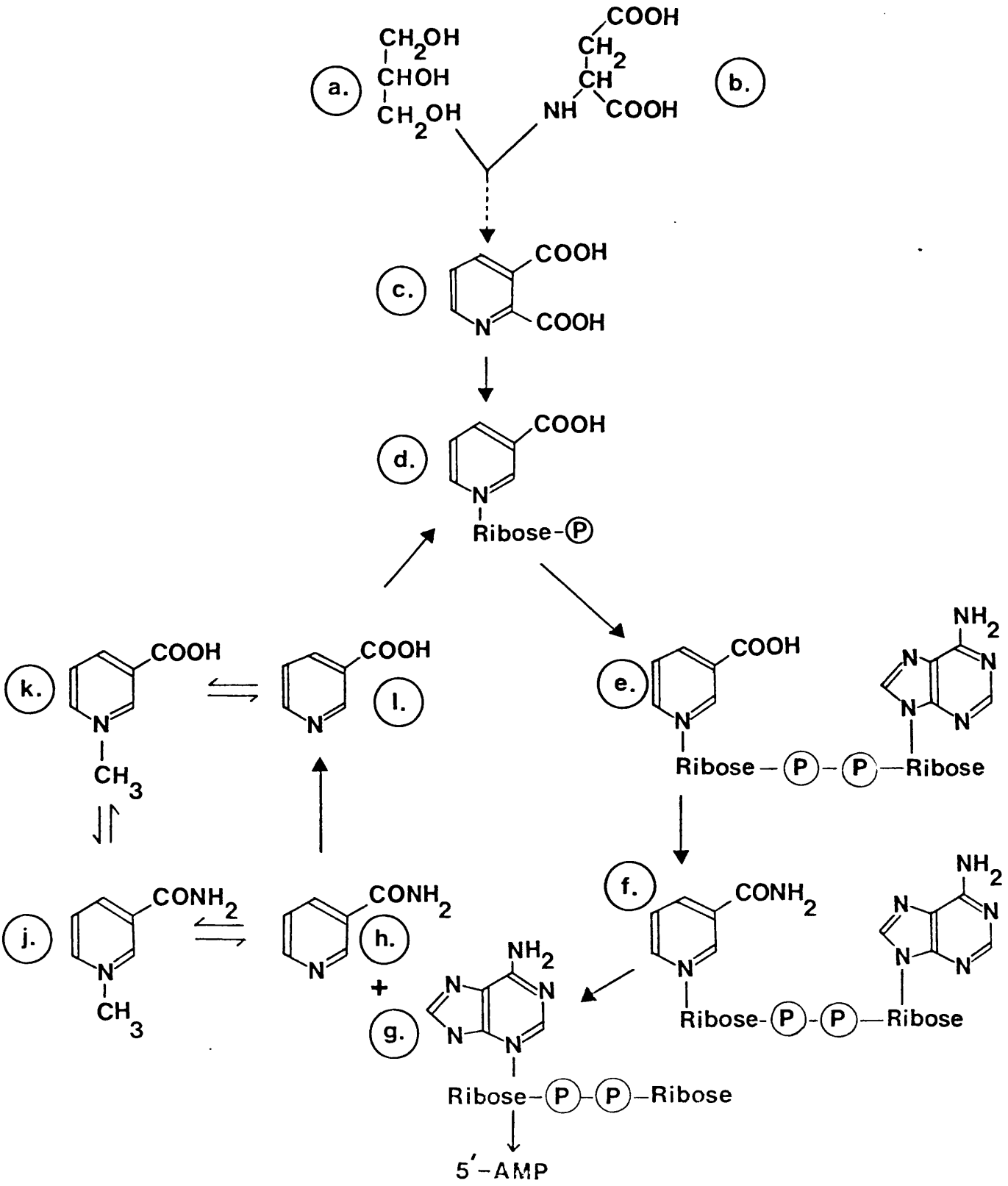
Fig. 1.3. NAD metabolism in plants: the Preiss-Handler pathway

Key:-
a. Glycerol.
b. Aspartic Acid.
c. Quinolinic Acid.
d. Nicotinic Acid Mononucleotide.
e. Nicotinic Acid Adenine dinucleotide.
f. Nicotinamide Adenine dinucleotide (NAD).
g. Adenosine Diphosphate Ribose (ADPR).
h. Nicotinamide.
j. N-methyl Nicotinamide.
k. N-methyl Nicotinic Acid.
l. Nicotinic Acid.

N.B.1. N-α-L- Arabino-Nicotinamide derived from N-Methyl Nicotinamide.

N.B.2. ADPR may be derived from one or both of:-

(i) via NAD glycohydrolase.
(ii) via Poly (ADP-Ribose) Polymerase followed by Poly (ADP-Ribose) Glycohydrolase.


a.
b.
c.
d.
e.
f.
g.
h.
j.
k.
l.
Ribose-P
Ribose-P-P-Ribose
5′-AMP

depending upon the species of cell used. Cultures of legume cells (e.g. mung bean, soybean, and garbarrzo bean) accumulated N-methyl nicotinamide, and umbelliferae cells (e.g. parsley) accumulated N-α-L-arabino-nicotinamide. The same group have also shown that the adenine moiety of NAD is degraded in cell cultures via hypoxanthine, xanthine, allantoin and allantoic acid, with an accumulation of the latter two compounds.

Prior to the commencement of this study there were only two reports which suggested that NAD in higher plants became involved in ADP-Ribosylation of acceptor molecules within the cell (see above). (Payne and Bal, 1976, Lin, 1976).

Several workers have investigated the changes in levels of nucleotides and nucleosides during germination. Brown (1965, 1967) has shown that during the first 40 hours of germination of pea that the AMP and ADP levels fell, whilst the ATP level initially rose, then fell again. Obendorf and Marcus (1974) have shown that there is a 10 fold increase in the ATP level of germinating wheat embryos within one hour of the onset of germination. However, most work of this nature appears to be undertaken in an attempt to be able to predict seed vigour (e.g. Ching, 1973) and the biochemical significance of these changes has yet to be elucidated.

The NAD content of various cereals has been examined:- in germinating wheat and oats (Bevilacqua, and Scotti,1953, Bevilacqua, 1955) and in germinating rice seeds (Mukherji et al, 1968). It was shown that the overall NAD level increased rapidly, however the rate of turnover was not measured. Using germinating peanuts Reed (1970) has shown that at the onset of germination there is a significant conversion of NAD to NADP,

with a coincident rise in NAD kinase, suggesting a possible active role for these compounds during germination.

Imbibition and subsequent germination cause several changes in the nucleic acid content of seeds, and have been studied extensively, primarily using maize seeds(Ingle et al, 1964). A significant part of the apparent redistribution of DNA and RNA is probably due to hydrolysis of the nucleic acids in the storage organs of seeds, coupled with re-utilisation of the purine and pyrimidine metabolites by the 'salvage' pathways which play a significant role in plant tissues during the early stages of germination (Shuster, 1963, Ross et al, 1971, Ashura, 1977).

Using isolated wheat embryos appreciable DNA synthesis has been known to start after approximately 12 hours of germination (Mary et al, 1972). It has also been found that DNA isolated from germinated embryos differs from that isolated from ungerminated embryos (Chen and Osborne, 1970), suggesting that modification of the DNA is occurring. The enzymes connected with DNA snthesis in higher plants appear to have very similar properties to those found in animal and bacterial systems, and several have been studied in detail by Harland et al (1973) in synchronous plant cell cultures. An overall view of nucleic acid metabolism in higher plants can be found in the work by Bryant (1976).

2. MATERIALS AND METHODS

2.1. Selection of Material

Wheat (Triticum Aestivus l.) seeds were obtained from Wiltshire Farmers Ltd. of Melksham, Wiltshire. Three different varieties, "Atou","Bouquet", and "Flanders", each with differing requirements for trace elements and germination conditions, were initially examined. The seed was supplied in 50 kg. sacks, pretreated with I.C.I. 'Mergamma', a combination treatment of γ- BHC and organic mercury compounds. The seeds were stored dry, in the dark at 0^{o} - $4^{o}C$, until used.

The germination of the different varieties of seeds under laboratory conditions was examined by placing seeds on moist filter paper and incubating at $26^{o}C$ in the dark. Germination was deemed to have occurred if both roots and a coleoptile were visible.

The effect of prewashing the seeds to remove the 'Mergamma' was also examined. The yield of root material was compared for each seed variety.

2.2. Preparation of Nuclei from Root Tips

It was decided to examine root tips, in the first instance, as these offered a readily available, rapidly dividing source of plant cells which were not protected by the very strong cell wall of most plant cells.

Intact seeds were germinated on a piece of moist filter paper, resting on a bed of glass beads(approximately 4 mm in diameter). The glass beads were immersed in water. This system had two advantages, firstly it prevented the seeds from being too wet (and therefore inhibiting their germination) and

secondly it allowed the roots to grow down and form a compact mat around the glass beads, which aided harvesting of the root tips. The germinations were carried out in the dark at 26°C in closed plastic boxes. Root tips (and coleoptiles if required) were harvested by hand.

The root tips (and coleoptiles) were homogenised in extraction buffer, 0.25M Sucrose, 0.01 M $MgCl_2$, 0.05M Tris-H Cl, 0.001%(v/v)Triton X-100, pH 7, (Icke, 1976), using a hand-held Potter-Elveshiem homogeniser. Both glass/glass and PTFE/glass homogenisers were examined. The resulting homogenates were stained with either Acetocarmine or Methylene Blue, both specific histological stains for nuclear material, examined under a high-power light microscope, and counted using a Neubaur Haemocytometer.

2.3. Preparation of Chromatin

The method employed was based on that of Simon and Becker (1976) as used with wheat germ. All operations described were carried out at 0° - 4°C. Approximately 25g of seeds were homogenised in 100 ml of TBT buffer (0.5% (w/v) Triton X-100 in 0.05M Tris-H Cl, pH 8.1, 15 mM β-mercaptoethanol) for 10 minutes using a Sorval Omnimixer. The homogenatewas washed out into a beaker with a further 20 ml TBT buffer, and stirred for 10 minutes. This extraction step was repeated several times until sufficient material had been prepared, then the homogenates were bulked together. The homogenate was filtered through two layers of butter-muslin, centrifuged in an MSE. 18 centrifuge at 4000g for 5 minutes and the precipitate discarded. The supernatant was made 0.05M with respect to $(NH_4)_2\ SO_4$, by dropwise addition

of 3M $(NH_4)_2\ SO_4$, and centrifuged at 10,000g for 10 minutes. The resulting supernatant was discarded and the pellet resuspended in 50 ml TBT buffer which was 0.05M with respect to $(NH_4)_2\ SO_4$. This suspension was then centrifuged at 10,000g for 10 minutes and the resultant pellet washed by resuspending in 50 ml. TBM buffer (0.01M Tris-H Cl, pH 8.1, 15 mM β-mercaptoethanol, 2 mM $Mg\ Cl_2$) and centrifuging at 10,000g for a further 10 minutes. This last step was repeated four times, the final pellet being a crude chromatin preparation.

When the chromatin was required for the preparation of histones (see below) the final pellet was made up in 1.7M sucrose, 15 mM β-mercaptoethanol, 0.01M Tris-H Cl (pH 8.1) and centrifuged at 60,000g for 2 hours. This pellet was washed by two cycles of suspension in TBM followed by centrifugation at 15,000g for 10 minutes.

2.4. Preparation of Viable Embryos from Intact Wheat Seeds

Intact, viable embryos were isolated after the method of Johnston and Stern (1957), which was modified to suit local conditions. The procedure adopted was as follows:-

Approximately 100g of intact dry seeds were mixed with small (about 1 cm^3) fragments of dry ice and homogenised in a 5l. stainless-steel Waring blender for one minute. The homogenate was then passed through a series of brass sieves (Endecott) of decreasing mesh size, 1.70 mm, 1.18 mm and 600 μm respectively. Embryos, plus similarly sized fragments of endosperm etc. were retained on the 600 μm sieve. The material retained on the 1.7 mm sieve was homogenised twice more. The entire procedure was repeated until sufficient

material was available for the next step.

Two alternative methods of separating the embryos from the other material retained on the 600 μm sieve were examined:-

(a) The retained material was stirred into 2M sucrose and the embryos allowed to float to the surface, the remainder of the seed fragments sank. The intact embryos were scooped off the surface of the sucrose solution, washed with ice-cold water, blotted dry then allowed to air-dry.

(b) The second method entailed stirring the retained material into cyclohexane: carbon tetrachloride (10:25 (v/v)). Again the embryos floated to the surface and were scooped off. At this stage two alternative methods of further treating the embryos were examined. The first was to wash and dry as described for method (a), the second was to simply air-dry the embryos.

Once the embryos had been dried most of the loose chaff isolated with them was blown off, by using either a compressed air line or a hairdryer. The embryos were then stored dessicated at $4^{o}C$ until required.

The viability of the isolated embryos was tested using two different techniques. First, the embryos were placed on a filter disc in a petri dish and moistened with germinating medium (1% (w/v) glucose, 0.01% (w/v) streptomycin sulphate) and incubated in the dark at $26^{o}C$. Second the embryos were placed on agar dishes (1% (w/v), Oxoid No. 1., 1% (w/v) glucose, 0.01% (w/v) streptomycin sulphate) and incubated in the dark at $26^{o}C$. Germination was judged to have occurred if roots were visible. The germination success of the embryos was checked at regular intervals throughout the study, using agar plates, 50 embryos to a plate.

2.5. Preparation of Nuclei from Viable Embryos

All preparative operations were performed at 0-4°C. Approximately 0.5g embryos were homogenised in 0.1M Triethanolamine (TEA) - H Cl pH 8.2, using a hand-held Potter PTFE/glass homogeniser, with approximately 15 ml. of buffer. The supernatant was filtered through two layers of butter-muslin. This last step was repeated until no more intact embryos were visible. The homogenate was centrifuged using an MSE bench centrifuge at 110g for 2 minutes, the precipitate discarded and the supernatant recentrifuged in the bench centrifuge at 2,700g for 15 minutes. The resulting pellet was the crude nuclear preparation. The presence of intact nuclei was observed using light microscopy as described in section 1.2. The pellet was made up to an appropriate volume, using 0.1M TEA - H Cl, pH 8.2 and was usually sonicated using an Ultrasonics Rapidas sonicator (4.2.).

2.6. Scintillation Counting

All radioactive samples were counted by liquid scintillation counting using a Packard Tri-Carb liquid scintillation counter. Two different scintillants were employed, one for non-aqueous samples and one for aqueous samples. For non-aqueous samples the scintillant used was 0.5% (w/v) 2, 5 - diphenyl oxazole (PPO) in redistilled toluene (PPO/Toluene). The scintillant used for aqueous samples was 0.5% (w/v) PPO, 30% (v/v) Triton X-100, 70% (v/v) redistilled toluene. The Triton/toluene/PPO scintillant will accept up to 10% final volume as water. Normally 2.0 ml of scintillant was used, the exception being some aqueous samples of low specific

radioactivity which therefore needed larger samples. When Millipore GF/C filters were used (see 2.7. below) the dry discs were placed on the side of the scintillation vials, with their lower edge immersed in the scintillant. When Whatman No. 1 2 cm paper discs were used they were placed face uppermost on the base of the scintillation vials. The counting efficiencies were obtained for each system used.

2.7. Estimation of Acid Insoluble Radioactivity

Two similar methods were employed, the first involved adding an aliquot of the reaction mixture (containing the acid insoluble radioactivity) to approximately 4 ml. of 20% (w/v) trichloroacetic acid (TCA), and allowing it to stand for 30 minutes on ice. This was then filtered onto Millipore GF/C discs, using an ultrafiltration tower, and the discs were each washed once with 10 ml. of absolute ethanol and then dried in an oven at 98^{o}C for 10-15 minutes. The discs were then ready for counting (see 2.6.).

The second method used Whatman No.1. 2 cm paper discs which had been pretreated by soaking them in 5% (w/v) TCA in diethyl ether for 30 minutes (approximately 75 ml. per 100 discs), removing the discs from the ether and allowing to air dry.

Up to 20 µl of reaction mixture was applied to each disc, which was numbered on its uppermost face with a soft (4B) pencil. The discs were then washed batch-wise (using approximately 75 ml. of washing medium per 100 discs), three times in 5% (w/v) TCA, once in absolute ethanol or methanol, depending upon availability, and finally in diethyl ether. Each washing treatment was for 15-30 minutes, and all were performed on ice.

When the discs had been removed from the ether and air-dried they were ready for counting.

2.8. DNA Estimation

The DNA estimations in this study employed the method of Setaro and Morley (1977), summarised below. The procedure used was to take 0.1 ml. of DNA containing solution and add to it 0.1 ml. 1.0 M H Cl O_4, add 0.1 ml. 1.32 M 1, 3, diamino-benzoic acid dihydrochloride (DABA) then incubate at 60°C for 20 minutes. The samples were then diluted with 1.7 ml. 0.5 M H Cl O_4 and the optical density read against a reagent blank at 470 nm. The standards for this assay were prepared by dissolving highly polymerised calf thymus DNA in 1 M NH_4OH (2.5 mg ml^{-1}) then placing suitable aliquots, to give a range 25-1000 ug DNA, in glass tubes and drying in an oven overnight at 70°C. The tubes were then stoppered and stored dessicated at 4°C until used. When required 0.2 ml. 0.5 M H Cl O_4 was added to each tube, followed by the DABA solution.

2.9. Preparation of [^{3}H] - Poly (ADP-Ribose) in vitro

In order to obtain sufficient acid insoluble cpm per assay to estimate poly (ADP-Ribose) degradation by wheat nuclei it was decided to synthesise [^{3}H] poly (ADP-Ribose) in vitro. Two methods were employed:-

(a) Using salt extracted pig thymus nuclei (gift of J. Whish). The salt extracted pig thymus nuclei were incubated at 25°C for 30 minutes in the presence of [^{3}H] -NAD. The mixture was made 20% (w/v) with respect to TCA by the addition of 60% (w/v) TCA solution, and allowed to stand on ice for 60 minutes. The

solution was then filtered onto a millipore GF/C filter disc, and washed with two volumes of 10% (w/v) TCA followed by one volume of absolute ethanol. The disc was air-dried, placed in approximately 5 ml. of 0.01 M Tris-H Cl, pH 7.6, containing 1mg ml^{-1} Pronase (E.C.3.4.24.4.) and incubated at $37^{o}C$. The amount of radioactivity in the solution was estimated by removing 50 µl of solution at intervals and counting direct using Triton/Tolene/PPO scintillant (see 2.6.). The incubation was continued until no more radioactivity was released into the solution. The supernatant was then removed and made 20% (w/v) with respect to TCA and stored overnight at $4^{o}C$. Next day the TCA was removed by repeated washings of diethyl ether, until the pH of the aqueous phase was approximately pH 7 - as judged using indicator paper. The aqueous phase was then split into aliquots and stored frozen until required.

(b) The second method used cultured HeLa cell nuclei which had previously been incubated with $[^{3}H]$ NAD (gift of P.R. Stone). The nuclear pellet, in 5% (w/v) perchloric acid (PCA) was dissolved in 0.1 M NaOH (approximately 3 ml.) and incubated at $37^{o}C$ for 3 hours. Once the incubation was complete an equal volume of water, 0.1 volumes of 1.0 M H Cl and 0.1 volumes of 0.5 M Tris H Cl pH 8.0 were added. The whole was then made 5 mM with respect to Mg^{2+} by dropwise addition of a 2.0 M $MgCl_2$ solution. Sufficient DNAse I (E.C.3.1.4.5.) was added to give a final concentration of 2mg. ml^{-1} and incubated for 3 hours at $37^{o}C$. At the end of this time an equal volume of dispersing buffer (0.5% (w/v) SDS, 0.15 M Sodium chloride, 0.015 M Sodium citrate, 0.05 M Tris pH8) was added and then pronase to a final concentration of 1 mg. ml^{-1} and allowed to

stand overnight at room temperature. A saturated phenol reagent was prepared by mixing phenol and water (approximately 92% (w/v) phenol), shaking and allowing to separate out, the lower phase being the phenol reagent.

The $[^3H]$ -label containing solution was mixed with an equal volume of phenol reagent, and left shaking gently at room temperature for 24 hours. Next day the aqueous phase was removed and re-extracted with phenol reagent for a further 2 hours. The aqueous phase was then extracted with 4 x 1 volumes of water-saturated diethyl ether, and the excess ether gently blown off. It was then made 0.2 M with respect to potassium acetate, by dropwise addition of a 0.5 M potassium acetate solution. Three volumes of absolute ethanol were then added and the solution stored overnight at -20°C. The following morning the sample was allowed to warm up gradually in an ice bath and then centrifuged at 12,000g for 15 minutes at 4°C. The resulting pellet was washed once with ice-cold absolute ethanol, resuspended in water (approximately 500,000 c.p.m. per ml.) and aliquots stored at -20°C until required.

2.10. $[^3H]$ NAD

The $[^3H]$ NAD employed in this study has been synthesised in this laboratory from $[^3H]$ ATP using the method of Ohtsu and Nishizuka (1971), and was at least 99% pure, as estimated by thin layer chromatography. The $[^3H]$ -label was in the adenine moiety of the NAD molecule at the 2 position and the specific radioactivity of the $[^3H]$ NAD was 20 m Ci. μM^{-1}; 1 mCi. ml^{-1}.

2.11. Thin Layer Chromatography

All thin layer separations were carried out on "Baker-Flex" PEI-cellulose t.l.c. sheets, 20 x 20 cm. Before use the plates were soaked in 2.0 M NaCl for 30 minutes, washed with distilled water, then soaked in distilled water for at least 45 minutes, and then air-dried. When dry the plates could be marked without damaging the PEI surface using a very soft (4B) pencil.

Material for the t.l.c. was invariably in 50% (v/v) ethanol, and was applied to the plate using capillary tubes, drying under a stream of cold air. Appropriate standards were always run with, and separately from, the experimental sample. The standards were normally 10 mg. ml^{-1} in 50% (v/v) ethanol and approximately 100 µg per cm. was applied to the t.l.c. sheet. The spots on the t.l.c. sheet were visualised under ultra-violet (U.V.) light. Three separate solvent systems were employed as given below.

System I

Used for separating ATP, ADP, AMP/ADPR and NAD. The plate was initially eluted for 3 cm. with 0.2 M LiCl, the t.l.c. sheet was then immediately transferred to 1.0 M LiCl and the sheet eluted for the remaining 13 cm.

System II

Used for separating PRAMP, ADPR, AMP, adenosine (Ado) and poly (ADP-Ribose). The system consisted of eluting the plate 2 cm with 1.0 M CH_3CO_2H then immediately transferring the sheet to a 9:1 (v/v) mixture of 1.0 M CH_3CO_2H and 3.0 M LiCl, and eluting the remaining 13 cm. (Rickwood *et al*, 1977).

System III

Used for separating adenosine (Ado) and ribosyl adenosine. Only non-polar compounds were eluted with this system, which consisted of eluting the sheet with double-distilled water. (Miwa et al, 1979a).

After the sheets had been removed from the eluants and dried (using a hairdryer) the spots were visualised under U.V. light and their position drawn on the sheet. At this stage one of two procedures was adopted. Either the whole spot was cut out and the PEI scraped off into a scintillation vial, or the track was marked into 0.5 cm strips, each was carefully cut out, and each strip scraped off into scintillation vials. In either case the subsequent procedure was common: 0.1 ml of 1.6 M Li Cl was added to each vial and allowed to stand for 30 minutes, then 1.9 ml Triton/Toluene/PPO scintillant was added and the samples counted for c.p.m. corresponding to that particular spot/strip.

2.12. Descending Paper Chromatography

Used to attempt to separate AMP, adenosine and ribosyl adenosine. The solvent used was 80% (v/v) ethanol, the paper length was 46 cm, and the running time 12 hours. When the paper had been dried and the spots visualised under U.V. it was cut into 1 cm strips, each strip placed in a scintillation vial, and 0.4 ml of 0.1 M H Cl was added. The vials were incubated at 37^{o}C for 30 minutes then 3.6 ml Triton/Toluene/PPO scintillant added and the samples counted.

2.13. Chain Length Determination

The principle of using a snake venom phosphodiesterase

(SVPDE) (E.C.3.1.4.1.) digestion of labelled poly (ADP-Ribose) and the subsequent separation of the 5' AMP and PRAMP by t.l.c. methods are well known (Nishizuka et al, 1969). This study used a slightly modified procedure as outlined below:-

The sample under investigation was precipitated overnight at -20°C in 90% (v/v) ethanol. The sample was then centrifuged using a Beckman microfuge at 10,000 g. for 5 mins at room temperature. The pellet was resuspended in 1 ml. of 100% ethanol and the sample spun again as above. An aliquot of the supernatant was counted direct, using Triton/Toluene/PPO scintillant (see 2.6.). This step was repeated until no ethanol-soluble c.p.m. were present in the supernatant. The pellet was then dissolved in buffer (0.05 M Tris H Cl, 0.01 M $MgCl_2$, 0.02 M glucose-1-phosphate, 4 M urea, with the pH adjusted to pH 8.8 using conc. H Cl) containing sufficient SVPDE to give 0.1 units of enzyme per assay. The SVPDE used was that supplied by Sigma. The sample was incubated at 37°C for 4 hours, and then the reaction was stopped by making the reaction mixture 20% (w/v) with respect to T.C.A., and standing on ice for 30 minutes. The sample was centrifuged as above, and the supernatant extracted with water-saturated diethyl ether until the pH of the supernatant was pH 6 (as estimated using indicator paper).

A suitable aliquot containing 2000-3000 c.p.m. was applied to a PEI-cellulose t.l.c. plate (see section 2.11.), and when dry the urea was removed from the t.l.c. plate by a 4 cm elution in anhydrous methanol, followed by the total immersion of the plate in anhydrous methanol for 30 minutes. After the plate had been dried a standard solution containing AMP, ADPR, GMP and adenosine was applied, and the plate eluted using t.l.c.

System II. After elution the plates were cut into 0.5 cm strips and treated as described in 2.11. The GMP standard was used as a marker for PRAMP. The average chain length was obtained by taking the ratio of radioactivity recovered as AMP to the amount of radioactivity recovered as PRAMP plus AMP:-

$$\frac{\text{(c.p.m. PRAMP) + (c.p.m. AMP)}}{\text{(c.p.m. AMP)}} = \text{average chain length}$$

The chain length determinations were performed in duplicate and the results averaged. A more detailed analysis of the experimental errors was performed with five individual determinations using [^{3}H] poly (ADP-Ribose) synthesised *in vitro* using method 2.9.b. This analysis gave a variation of $\pm$ 3.7%.

The incubation with SVPDE was judged to have been complete because no radioactivity was detected on the origin of the t.l.c. plates (Fig. 2.1.). To check that the reaction went to completion in the time allowed, a separate experiment was performed. Using [^{3}H] poly (ADP-Ribose) synthesised from isolated HeLa nuclei (2.9.b.), aliquots were removed from the reaction mixture at various intervals and counted for acid insoluble radioactivity. The results are shown in Fig. 2.2. and show that the reaction rapidly proceeds to completion.

2.14. Histone Extraction

Histones were extracted from the crude nuclear preparations as follows:-

The preparation was made 0.2 M with respect to H_2SO_4 by the addition of 2M H_2SO_4 and stood on ice for 90 minutes, mixing occasionally. The sample was centrifuged using a Beckman microfuge at 10,000g for 5 mins and the supernatant stored on

Fig. 2.1. Representative t.l.c. profile from chain length determinations

Each strip was 5 mm wide, scraped off, then 100 µl 1.6 M Li Cl added followed by 2.0 ml Triton/Toluene/PPO scintillant, markers used were ADPR, GMP (runs with PRAMP) and 5' AMP.

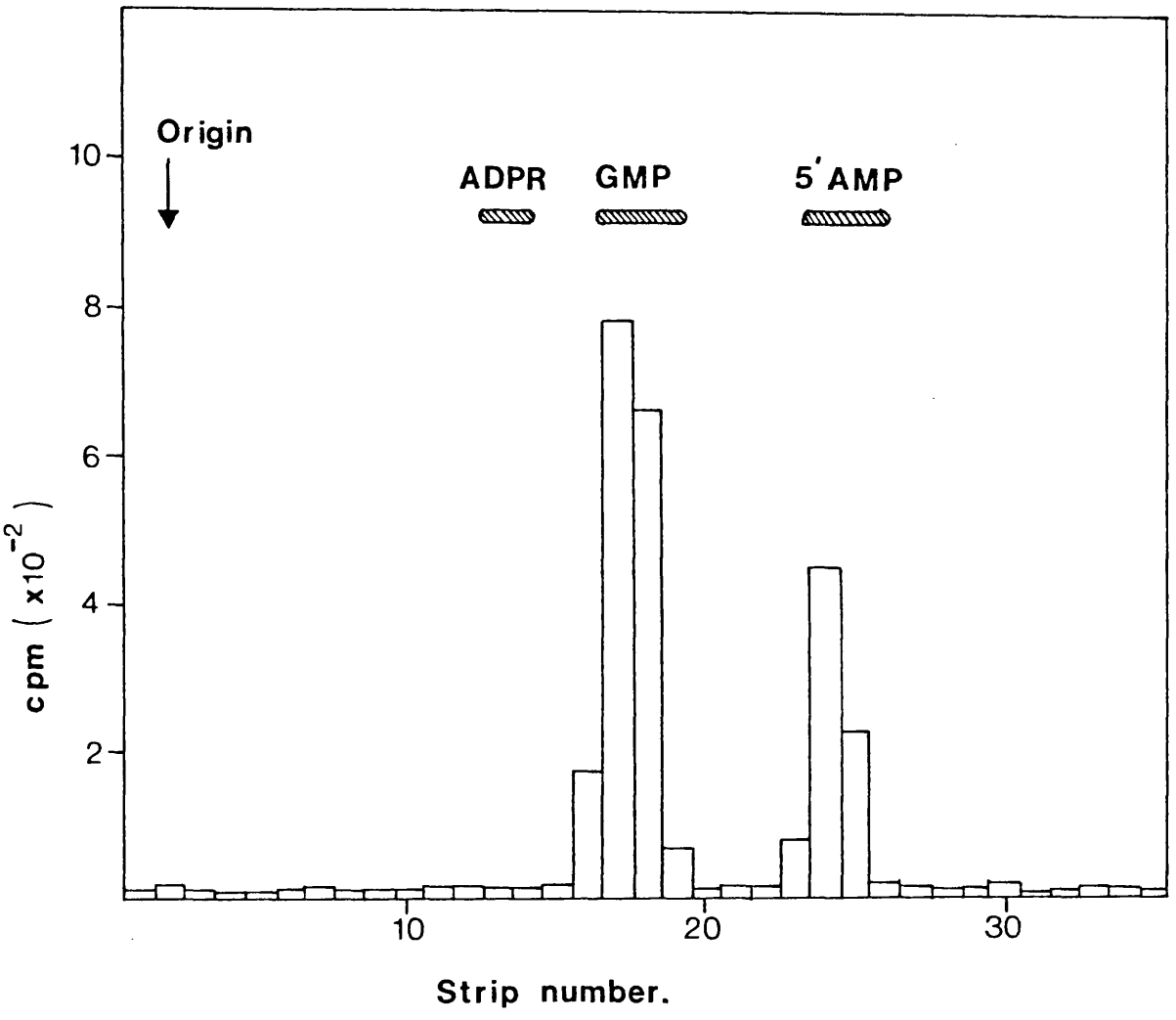
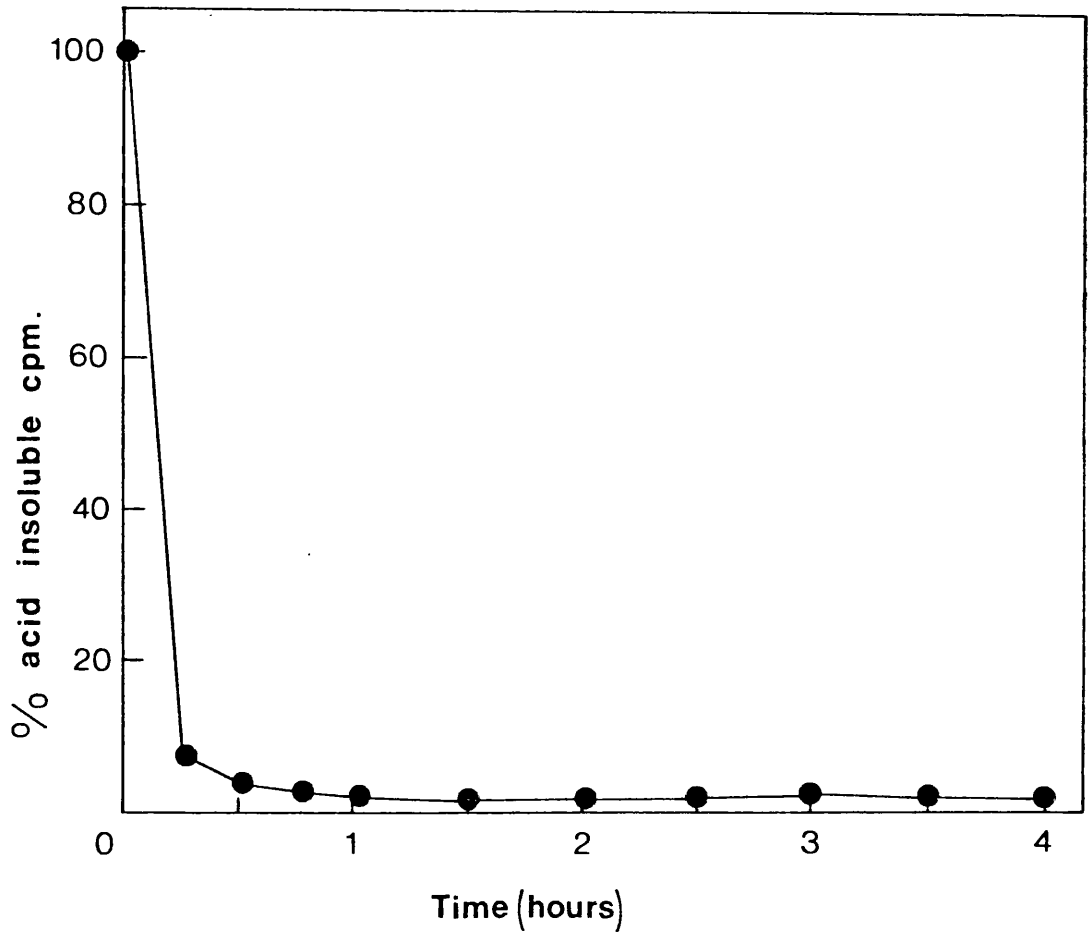

Origin
ADPR
GMP
5'AMP
10
8
6
4
2
cpm (x10^{-2})
10
20
30
Strip number.

Fig. 2.2. Time course of SVPDE digestion of [^{3}H] poly (ADP-Ribose)

Incubation carried out in 0.05M Tris-HCl, 0.01M $MgCl_2$, 0.02M Glucose-1-phosphate, 4 M urea, pH 8.8, 37^{o}C.


100
80
60
40
20
0
1
2
3
4
% acid insoluble cpm.
Time (hours)

ice. The pellet was re-extracted using 0.2M H_2SO_4 for 45 minutes on ice. The sample was then centrifuged as above and the two supernatants combined. A five-fold excess of 100% ethanol was added to the combined supernatant and stored overnight at -20°C. The following morning the sample was centrifuged as above and the pellet resuspended in 1 ml of ice-cold 20% (w/v) TCA, and recentrifuged as above. The pellet was washed in a like manner by two further 1 ml aliquots of 20% (w/v) TCA and two 1 ml aliquots of absolute ethanol. An aliquot of the final supernatant was checked to ensure that no ethanol soluble radioactivity was present.

This pellet was resuspended in a small volume of water, recentrifuged as above, and the resulting supernatant used as the soluble total histone preparation. Histone H1 was preferentially extracted from the total histone preparation by taking an aliquot and making it 5% (w/v) with respect to PCA by the addition of 72% (w/v) PCA, and standing on ice for 20 minutes. After centrifugation as above the supernatant contained histone H1. (Johns, 1964)

The resuspension of the pellets was assisted by the use of a 'Whirlimix' and a Dawe Instrument Ltd. Sonicator Bath.

2.15. Acid-Urea Electrophoresis

The method followed was that of Panyin and Chalkley (1969) using 6.25 M urea. The gels were prepared by mixing 4 ml of solution A (60% (w/v) acrylamide, 0.4% (w/v) bis-acrylamide in water) with 2 ml of solution B (43.2% (v/v) glacial acetic acid, 4% (v/v) TEMED) and 10 ml of solution C (10 M urea, 0.2% (w/v) ammonium persulphate). 1.1 ml of gel solution was placed in

each tube, overlaid with water, and allowed to polymerise (approximately 25 minutes).

A 50 µl aliquot of histone preparation (see 2.14.) was mixed with an equal volume of buffer (30% (w/v) sucrose, 1.8M CH_3CO_2H, 0.1% (w/v) methylene blue) and placed on top of the gel after the overlay of water had been removed. The buffer used in each reservoir of the apparatus 0.9M CH_3CO_2H, the upper reservoir being the anode. The gels were run at a constant current of 2 mAmp per tube at room temperature until the methylene blue marker was within a few mm of the bottom of the gel. The gels were removed from the glass tubes and left overnight in stain solution (2.5g coumassie blue 'G', 454 ml methanol, and 92 ml glacial acetic acid, made up to 1 l. with water) or 0.9M CH_3CO_2H depending upon their subsequent use. Excess stain was removed by destaining in 0.9M CH_3CO_2H at 50^{o}-$60^{o}C$, changing the destaining solution at frequent intervals until the protein (histone) bands were clearly visible. These gels were then scanned at 620 nm using a Joyce-Loebl Chromoscan.

Duplicate gels were frozen on dry ice and sliced. Each slice was placed in an empty scintillation vial and left overnight at $37^{o}C$. The following morning 100 µl of water and 500 µl of Soluene ((Packhard) were added, the vials tightly capped and stored at $50^{o}C$. After 24 hours 10 ml of Triton/Toluene/PPO scintillant (2.6) were added and the vials stored at $4^{o}C$ for 1 hour before counting for radioactivity.

3. RESULTS

3.1. Germination Studies

When whole seeds were germinated (2.1.) there was no difference in viability between the three varieties, 'Atou', 'Bouquet' and 'Flanders'. The yield of root tips from the germinated dry seeds was also examined. The following results are the yield in grammes wet weight per 100g dry seed. 'Atou' yielded 3.6±0.07g, 'Bouquet' yielded 4.01±0.1g and 'Flanders' gave 4.1±0.1g. As a result 'Flanders' was used for all further studies.

3.2. Isolation of nuclei

The root tips obtained from germinated intact seeds were homogenised and the resulting homogenates were examined for the presence of intact nuclei. No difference was observed when either glass/glass or glass/PTFE Potter-Elveshiem homogenisers were used. It was found that 1g (wet weight) of root tips yielded approximately 2.7×10^7 nuclei. The nuclei were clearly visible using both acetocarmine and methylene blue. The latter was preferred as it did not cause fragmentation of the nuclei. The homogenisation of the coleoptiles was found to be impractical as a source of nuclei owing to their fibrous nature and difficulties in the subsequent isolation of the nuclei. This method of isolating nuclei was abandoned because:

(a) it was difficult to grow a sufficient amount of seeds,

(b) the excessive time taken to harvest the root tips, and

(c) difficulties in ensuring that the germinating seeds were free of fungal contamination.

3.3. Isolation of Chromatin

Chromatin was prepared from intact dry seeds (2.3.). The chromatin was examined for [^{3}H] NAD incorporating activity (see below, 4.1.) and for histone analysis (see below, 6.6.). The chromatin preparations obtained proved suitable for polyacrylamide gel electrophoretic analysis but were not found to be suitable for enzyme characterisation. Preparation of the material took approximately 3-4 hours, the final pellet was difficult to dissolve and the resulting solutions were very viscous. This in turn led to sampling and assay difficulties.

3.4. Isolation of embryos

In an attempt to overcome the problems encountered using either nuclei from root tips, or chromatin from seeds, an alternative approach was tried. Intact wheat embryos were prepared as outlined in 2.4. Two purification methods were examined.

Method (a) was very slow, complete separation of the embryos from similar sized endosperm fragments took up to 2 hours. The embryos which were isolated were difficult to handle, and even after washing and blotting dry had a tendency to remain in solid lumps. Method (b) took approximately 5 minutes to give complete separation of the embryos from other seed fragments. When the embryos were subsequently washed and dried (as for method (a)) they also had a tendency to stick together, but when they were air-dried no such "clumping" was encountered.

Both alternatives gave viable embryos. Method (a) gave results varying between 50% and 90% viability for different preparations. Method (b), when used in conjunction with a final wash and blot dry, gave a viability of 75% to 95%. When the

embryos isolated by method (b) were air-dried the viability was between 95% and 100%.

The routine preparation of embryos employed method (b) coupled with air-drying. Embryos were discarded if their viability fell below 90%. Nuclei were then prepared from the viable embryos (see above, 2.5.). One gramme (dry weight) of viable wheat embryos yielded approximately 8×10^7 nuclei. When examined using a light-microscope the nuclear preparations appeared to be significantly free of debris. Further purification of the nuclei was not attempted for two reasons. Firstly was the time factor, a nuclear preparation as described above took approximately 45 minutes to prepare. Secondly, under the conditions employed, the nuclei were sensitive to osmotic shock and approximately 50% intact nuclei were lost in half an hour.

3.5. Scintillation Counting

The counting efficiency (see 2.6.) for 0.5% (w/v) PPO in Toluene was 42.09% using GF/C discs. When 2 cm Whatman No. 1 discs were employed the counting efficiency was 39.98%. When 0.5% (w/v) PPO, 30% (v/v) Triton X-100, 70% (v/v) Toluene was used for direct counting the efficiency was 22.04%.

3.6. In Vitro preparation of [^{3}H] poly (ADP-Ribose)

The method of preparing [^{3}H] poly (ADP-Ribose) from pig thymus (2.10a) was not very successful. After the [^{3}H] poly (ADP-Ribose) had been acid precipitated on to a GF/C disc only 36% of the acid insoluble radio-activity was solubilised when incubated with pronase for 24 hours. In addition, when GF/C discs were incubated for any length of time, they began to break up. This

led to subsequent sampling inaccuracies because of glass fibre fragments (to which the polymer adhered) in the [^{3}H] poly (ADP-Ribose) preparation.

The use of cultured cell HeLa nuclei as a source of [^{3}H] poly (ADP-Ribose) proved more satisfactory (2.9.b.). Polymer was synthesised by incubating isolated nuclei with [^{3}H] NAD. The yield of polymer was determined at each step of the purification procedure. The values given below have all been corrected to d.p.m. because two different counting systems were employed (see 3.5.). 12% of the initial d.p.m. were lost at the phenol extraction stage. 4% of the material was lost during overnight ethanol precipitation, and 21% lost during the ethanol washing stages, thus leaving 63%. The final pellet was readily soluble in water and was dissolved in water to give approximately 500,000 acid insoluble c.p.m. per ml. Aliquots (approximately 250 µl) were stored at -20^{o}C until needed. A chain length determination (see 2.13.) was performed on this preparation and an average chain length of 5.14 ± 0.16 was obtained. Digestion of the preparation with SVPDE and alkaline phosphatase (full details in 5.1.) gave rise to ribosyl adenosine and adenosine. Using t.l.c. system III 82% of the acid insoluble radioactivity was found to be converted to ribosyl adenosine, and the rest to adenosine. This is conclusive proof that the [^{3}H] label was present in poly (ADP-Ribose).

3.7. Polyacrylamide gel electrophoresis of Histones

The number and relative mobilities of the histone bands visible after the excess stain had been removed were in close agreement with published results for plant histones (Nadeau et

al, 1974). Once the gels were destained they were photographed and representative examples are given in Fig. 3.1. to Fig. 3.4. These figures show comparisons between histones isolated from nuclei, chromatin and preferentially extracted histone H1 from chromatin. As can be seen, pre-incubation of samples with NAD prior to electrophoresis does not lead to any visible new band (Fig. 3.3. and Fig. 3.4.) as might be expected from previous studies (Rickwood et al, 1977).

It is also apparent that several other nuclear proteins are co-extracted with the histones from wheat nuclei (Fig. 3.2.). It is also noticeable that the extraction of histone H1 is not completely selective (Fig. 3.1.). However, the results obtained were sufficiently clear to identify histone H1 in the various gels. Destained gels were also scanned at 620 nm; representative profiles can be seen in Fig. 6.3.

It proved impossible to both scan and then slice and count gels of material labelled with $[^3H]$ NAD. This was because the conditions used to destain the gels (50^o - 60^oC, 0.9M CH_3CO_2H) caused 67% loss of acid insoluble radioactivity in 90 minutes and 100% loss in 180 minutes. As the destain procedure routinely took 36-48 hours it was impossible to both destain and count the same gel. This problem was overcome by running duplicate gels. One gel was stained, the other was used for radioactive counting, and the two compared directly.

Using the techniques given above it was found that only histones H1, H2A and H2B are ADP-Ribosylated in vitro when wheat nuclei are incubated with $[^3H]$ NAD. Histone H3 may be modified, the results are not conclusive in this respect; no modification of histone H4 was observed. A full breakdown

of histone modification can be seen in Table 6.3.

Fig. 3.1. Comparison of histone H1 with total histones extracted from a chromatin preparation

A = Histone H1

B = Total histones


A
B
H1
H1
H2A/H2B
H3
H4

Fig. 3.2. Comparison of Histones extracted from isolated wheat nuclei with histones extracted from a chromatin preparation

A = Total histones from isolated nuclei

B = Total histones from a chromatin preparation

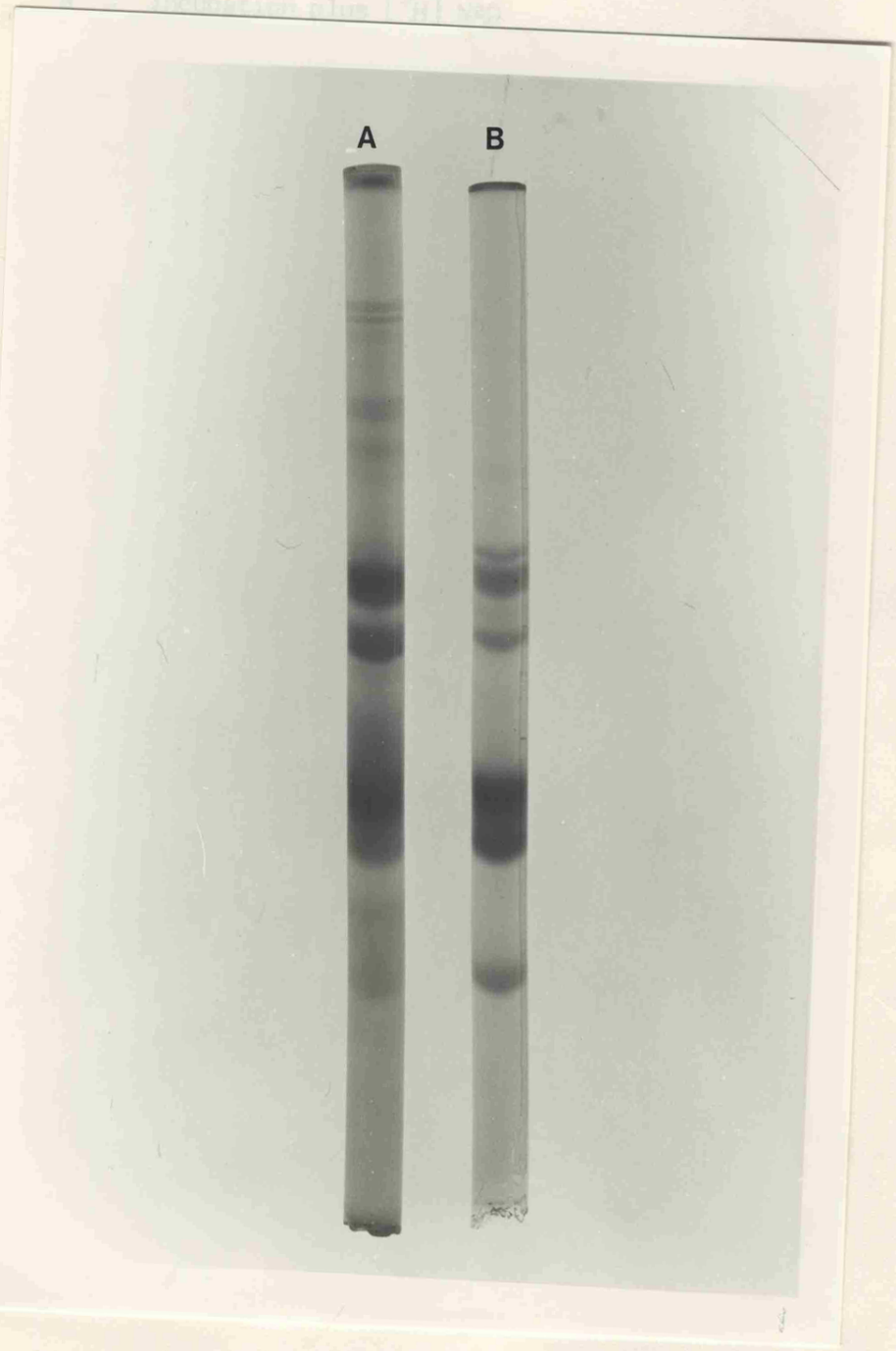

A
B

Fig. 3.3. Comparison of Histones extracted from isolated wheat nuclei incubated plus and minus $[^3H]$ NAD

A = incubation plus $[^3H]$ NAD

B = incubation minus $[^3H]$ NAD


A
B

Fig. 3.4. Comparison of Histones extracted from a chromatin preparation incubated plus and minus [^{3}H] NAD

A = incubation plus [^{3}H] NAD

B = incubation minus [^{3}H] NAD


A
B

4. POLY (ADP-RIBOSE) POLYMERASE

4.1. Preliminary Investigations

The initial aim of this study was to demonstrate the presence of poly (ADP-Ribose) in plants. The early experiments were performed using nuclei from root tips (see 2.2. above). When such nuclei were incubated with [^{3}H] NAD acid insoluble radioactivity could be seen. Formation of acid insoluble radioactivity was shown to be inhibited by nicotinamide and strong denaturing agents, e.g. 5% (w/v) TCA. Although the use of nuclei from root tips was abandoned (see above) further studies were continued using a chromatin preparation from intact seeds (2.3.). When the chromatin preparation was incubated under identical conditions incorporation of [^{3}H] NAD into acid insoluble radioactivity occurred. The resulting reaction mixture was subsequently treated with SVPDE and when the products analysed by t.l.c. (see 2.11.) PRAMP was identified. Since PRAMP is a unique product of SVPDE digestion of poly (ADP-Ribose) it was concluded that [^{3}H] NAD was incorporated into poly (ADP-Ribose) via poly (ADP-Ribose) polymerase. A number of other, unidentified compounds were also seen on the t.l.c. plate (Fig.5.1.). The incorporation of [^{3}H] NAD into poly (ADP-Ribose) was shown to be temperature dependent, with an optimum of 26.5^{o}C. Optimal activity was seen at pH8.0. An absolute requirement for reduced thiol groups was shown, since 5mM dithionitrobenzoic acid (DTNB) caused 98% inhibition of incorporation. In addition, nicotinamide and 3 amino benzamide (Purnell and Whish, 1980) were shown to have an inhibitory effect on poly (ADP-Ribose) synthesis. Further studies using nuclei from intact embryos showed identical characteristics to those outlined above using nuclei from

root tips or a chromatin preparation. The remainder of this study used nuclei from embryos.

4.2. Standard Assay Conditions

When a nuclear preparation was sonicated prior to incubation a 3-4 fold increase in incorporation of [^{3}H] NAD was seen, thus sonication was routinely used prior to all assays. Standard assay conditions were adopted, and were used throughout. These were using sonicated nuclei in 0.1M triethanolamine-HCl, 2mM $MgCl_2$, 10mM β mercaptoethanol, pH8.0, incubating with 2.5 μM [^{3}H] NAD at 26°C. Acid insoluble radioactivity was determined as described in 2.7. Where variations from this method were employed they are noted below as appropriate. Assay volume was 250 μl. (approx. 8×10^6 nuclei), incubated for 8 minutes.

4.3. Time Course of Incubation with [^{3}H] NAD

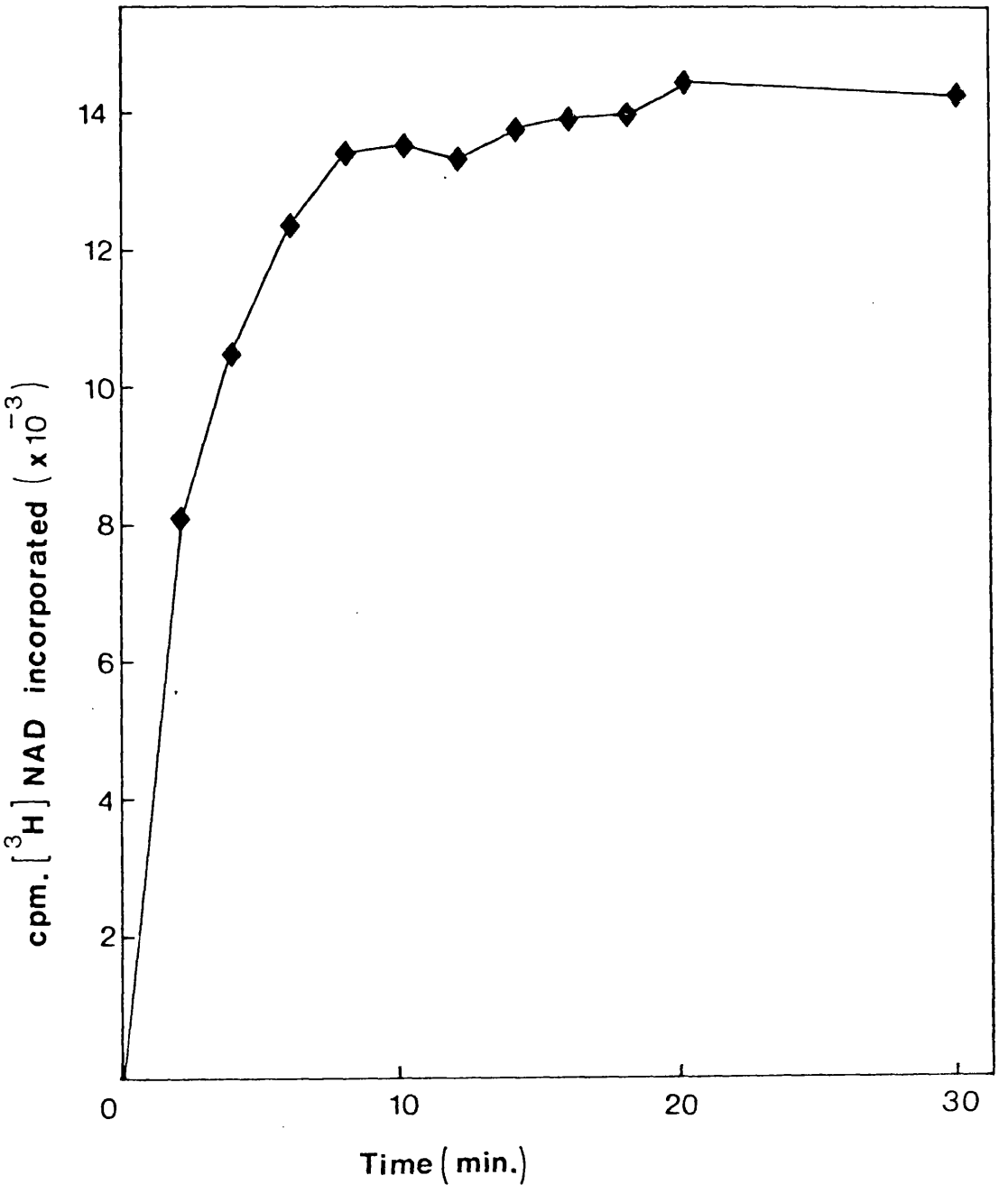
The incorporation of [^{3}H] NAD into acid insoluble radioactivity, using the standard assay conditions was examined. The reaction followed linear kinetics for approximately 10 minutes and thereafter plateauxed out. The results are shown in Fig. 4.1.

4.4. Characterisation of Products of [^{3}H] NAD Incorporation

The products of the standard reaction-mixture were analysed in an attempt to show the unambiguous presence of poly (ADP-Ribose). The reaction products were digested with SVPDE (full details are given in 2.13.). After digestion, aliquots were subjected to t.l.c. analysis, using the techniques given in 2.11. A compound corresponding to PRAMP was identified, but some problems were encountered due to the apparent ability of

Fig. 4.1. Time course of incubation of a preparation of isolated wheat nuclei with [^{3}H] NAD

Incubations carried out in 0.1M TEA-HCl, 2mM $MgCl_2$, 10mM β mercaptoethanol, pH 8.0, 26^{o}C.


14
12
10
8
6
4
2
0
10
20
30
cpm. [3H] NAD incorporated (x10-3)
Time (min.)

the nuclear preparation to further metabolise PRAMP (see Fig. 5.1.). The SVPDE digest was further hydrolysed with alkaline phosphatase (E.C.3.1.3.1.) and a compound corresponding to $[^3H]$ ribosyl adenosine was isolated. Digestion of poly (ADP-Ribose) with SVPDE will lead to the formation of PRAMP (plus AMP, which is derived from the terminal ADP-Ribose residue). Further digestion of the PRAMP, using alkaline phosphatase, will then give ribosyl adenosine. Using the t.l.c. system III (2.11.) it is possible to clearly distinguish between adenosine and ribosyl adenosine. Analysis of the final reaction mixture (after SVPDE and alkaline phosphatase digestion) using t.l.c. system III showed that the ratio of ribosyl adenosine to adenosine was 2.4:1. When a chain length determination (2.13.) was carried out a value of 2.51 ± 0.09 was detained. It was therefore concluded that formation of poly (ADP-Ribose) in vitro is catalysed by isolated wheat nuclei.

4.5. Temperature Dependance

The standard reaction mixture was incubated at different temperatures for 8 minutes, during which time the kinetics are linear (see Fig. 4.1.). Aliquots were removed and counted for acid insoluble radioactivity. The results can be seen in Fig. 4.2. and show an optimum temperature of 17^oC. An estimate of the decay rate constant of the polymerase at each temperature was obtained by calculating the rate of enzyme activity from the respective time courses. This decay rate constant was obtained by plotting log-rate vs. time. An exponential temperature dependance was observed which gave a linear Arrhenius plot, as shown in Fig. 4.3.a. From the equation $\log_{10}R = \log_{10}R_0 - Kt$

Fig. 4.2. Effect of varying temperature on the poly (ADP-Ribose) polymerase activity in isolated wheat nuclei

Incubations carried out in 0.1M TEA-HCl, 2mM $MgCl_2$, 10mM β mercaptoethanol, pH 8.0, (100% c.p.m. approx. 3,500)

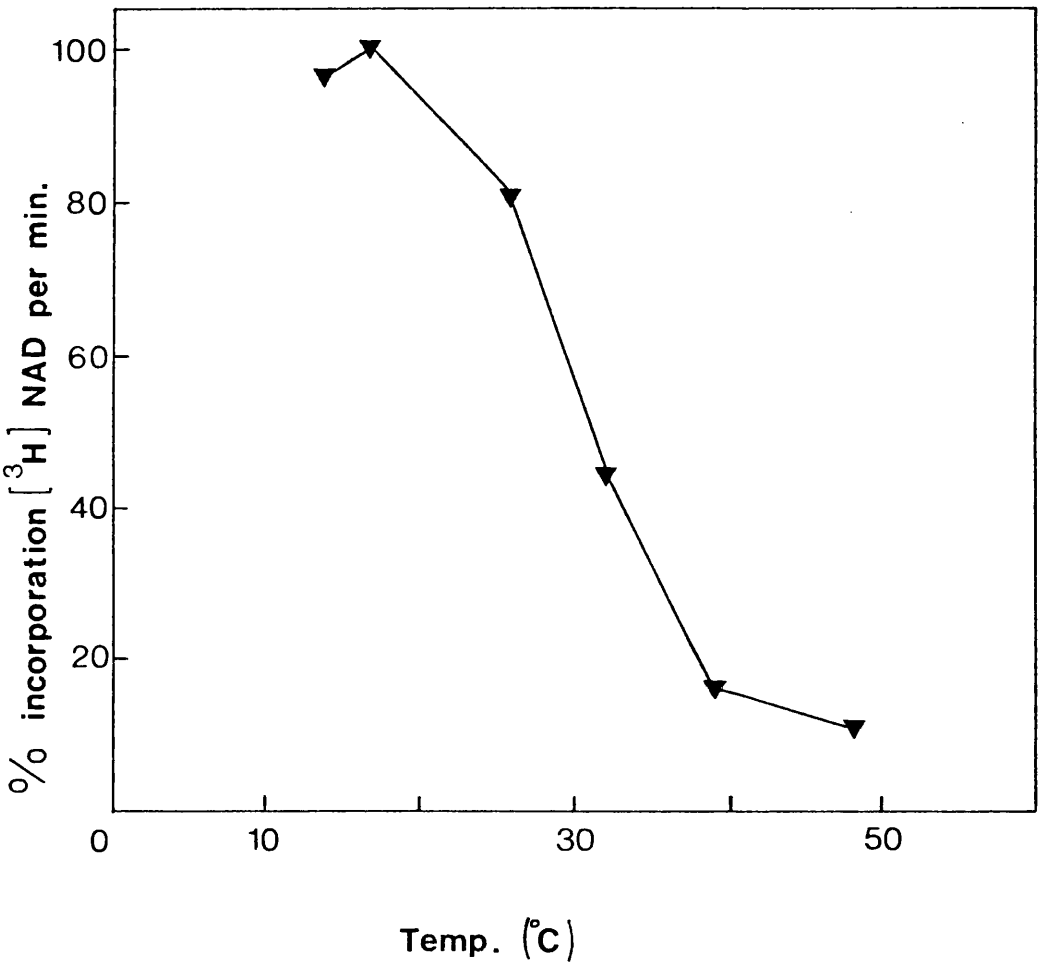

100
80
60
40
20
0
10
30
50
% incorporation [3H] NAD per min.
Temp. (°C)

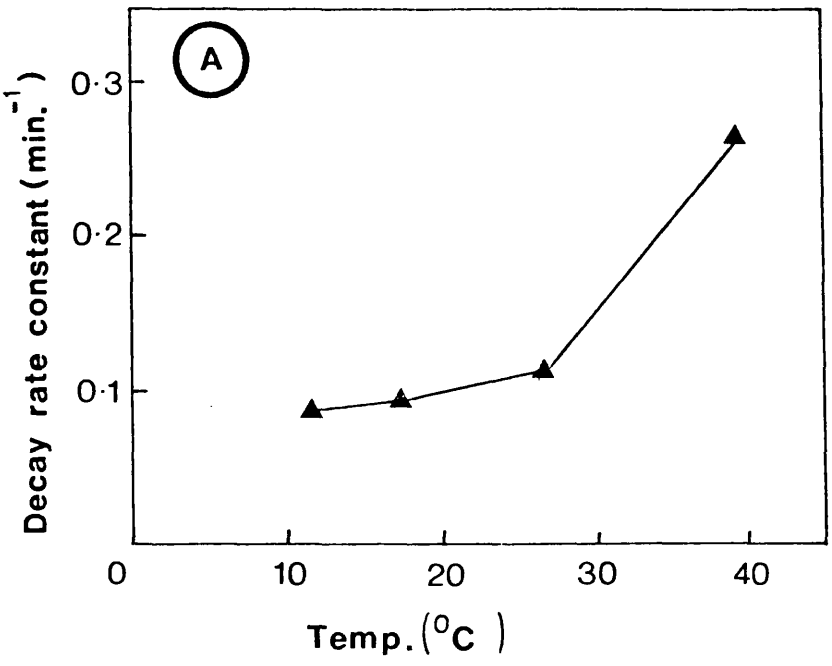
Fig. 4.3.a. Plot of decay rate constant of poly (ADP-Ribose) polymerase activity in isolated wheat nuclei at different temperatures

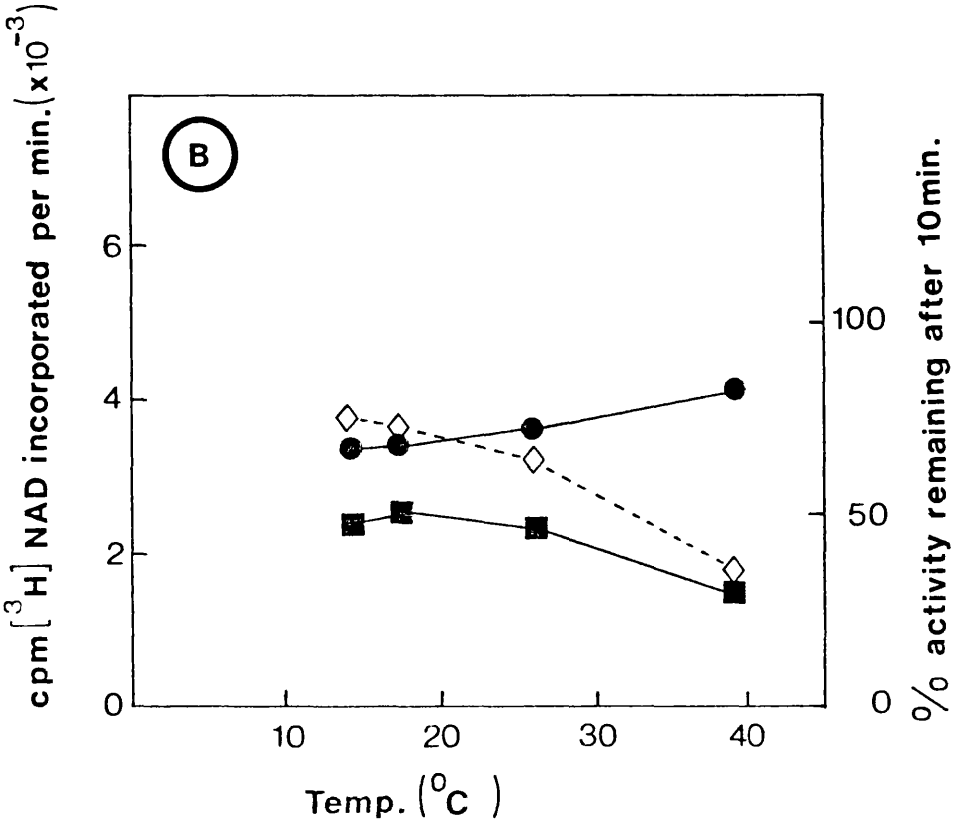
Fig. 4.3.b. Relationship between decay of poly (ADP-Ribose) polymerase activity and increase in initial rate

● = rate of activity at zero time

■ = rate of activity at 10 minutes

◇ = percentage activity remaining at 10 minutes


A
Decay rate constant (min.$^{-1}$)
0·3
0·2
0·1
0
10
20
30
40
Temp. (^{0}C)


B
cpm [^{3}H] NAD incorporated per min. (x10^{-3})
6
4
2
0
100
50
0
% activity remaining after 10min.
10
20
30
40
Temp. (^{0}C)

(where R = rate of activity at any given time t, R_0 = zero time rate and K = rate of decay) the calculated rate of enzyme activity at 10 minutes was obtained. The calculated rate of activity et 10 minutes was plotted against the zero time, as shown in Fig. 4.7.b. This figure also shows the percentage of activity remaining at 10 minutes. These results show a considerable degree of similarity to those of Stone and Shall (1973) who used isolated mouse LS 929 nuclei. It can be seen that the temperature dependance of the poly (ADP-Ribose) activity is the resultant of the opposing processes of enzyme decay and the increase in the initial rate of activity with temperature.

4.6. pH Dependance

The pH optimum of the poly (ADP-Ribose) polymerase was determined by taking aliquots of a nuclear preparation in 0.1M TEA-HCl, 2mM, $MgCl_2$, 10mM β mercaptoethanol pH 8.0 (as used in the standard assay) and mixing with either 0.1M citrate-phosphate or 0.1M Tris-HCl buffers at differing pH(Methods in Enzymology 1955). A 1:20 dilution of the nuclear preparation was made with the appropriate citrate-phosphate or tris-HCl buffer and the pH checked. Both the citrate-phosphate and tris-HCl were 2mM $MgCl_2$, 10mM β mercaptoethanol. The samples were incubated with $[^3H]$ NAD in the standard assay at 26°C. As can be seen from Fig. 4.4. the polymerase has a pH optimum of pH 7.9.

4.7. Requirement for -SH Groups

That the enzyme has thiol groups necessary for activity was shown by incubating the nuclear preparation with varying

concentrations of β mercaptoethanol and DTNB. The nuclear preparation was made using β mercaptoethanol-free buffer, the incubation was otherwise as standard. At the same time a sample was treated with 5 mM DTNB (final concentration). An optimum concentration of 10 mM β mercaptoethanol is needed for maximal enzyme activity (Fig. 4.5.). The effect of Hg^{2+} on the polymerase activity was also investigated by incubating a nuclear preparation with varying concentrations of Hg $(CH_3CO_2)_2$ under the standard assay conditions. It was found that 0.5 mM Hg^{2+} gave complete inhibition of the enzyme. The results are shown in Table 4.1.a. The observed loss of activity was investigated further to determine whether the Hg^{2+} inhibition was reversible. To test this a nuclear preparation was made and samples were preincubated with:- (a) buffer; (b) 20 mM β mercaptoethanol; (c) 1mM Hg $(CH_3CO_2)_2$; (d) 1mM Hg $(CH_3CO_2)_2$ with subsequent addition of 20mM β mercaptoethanol. These samples were then incubated with $[^3H]$ NAD as standard. Thus as can be seen in Table 4.1.b. 94% of the polymerase activity was recovered by treatment with β mercaptoethanol following Hg^{2+} treatment, indicating completely reversible Hg^{2+} inhibition.

4.8. Effect of Mg^{2+}

The optimal Mg^{2+} concentration for the enzyme was then determined using the standard assay procedure, but varying the final concentration of $MgCl_2$. The nuclei were prepared in $MgCl_2$ - free buffer. The results gave an optimum concentration of 2mM Mg^{2+} (Fig. 4.6.).

Fig. 4.4. Effect of varying pH on the poly (ADP-Ribose) polymerase activity in isolated wheat nuclei

Incubations carried out at 26°C, using either 0.1M Citrate-phosphate buffer (below pH 7.8) or 0.1M Tris-HCl buffer (pH 8.0 and above) as described in 4.6.

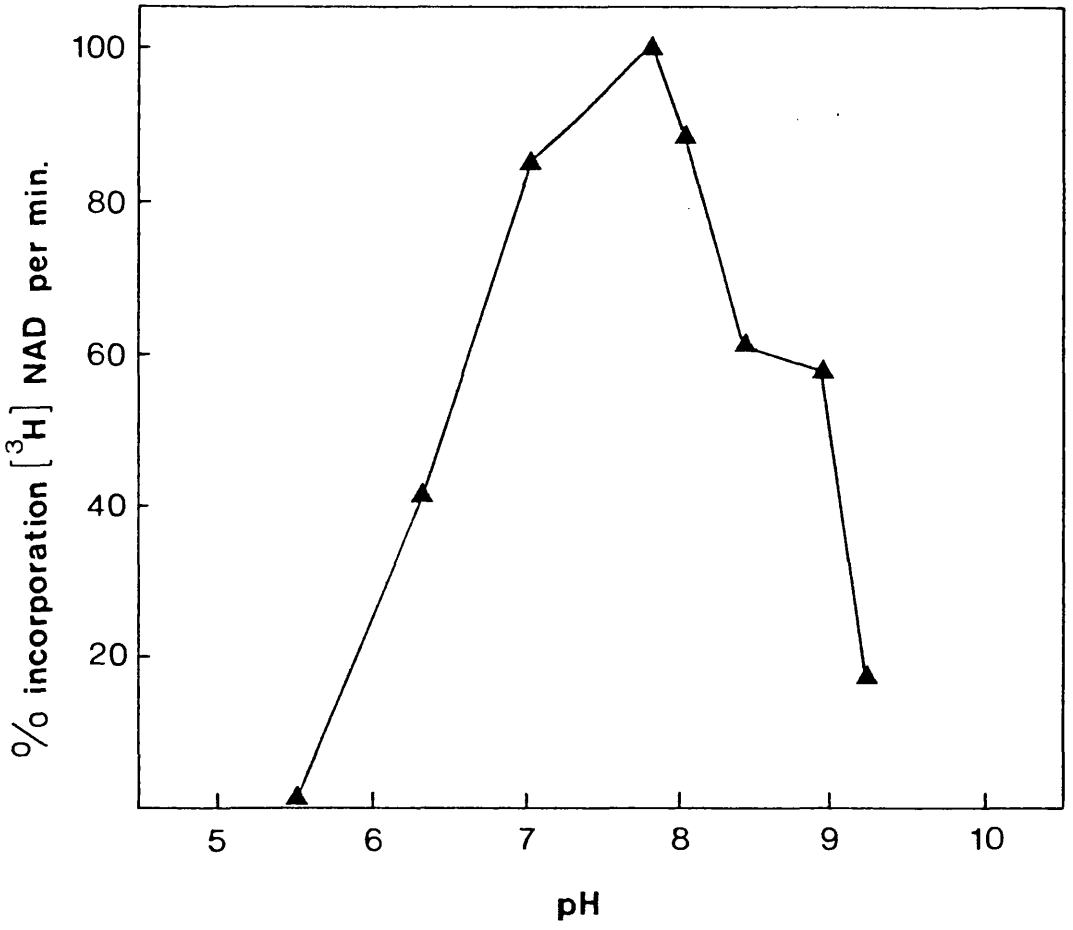

100
80
60
40
20
% incorporation [3H] NAD per min.
5
6
7
8
9
10
pH

Fig. 4.5. Effect of β mercaptoethanol and DTNB on poly (ADP-Ribose) polymerase activity in isolated wheat nuclei

○ = control (no β mercaptoethanol)

● = 1mM β mercaptoethanol

□ = 2mM "

■ = 3mM "

△ = 5mM "

▲ = 10mM "

◆ = 5mM DTNB

Incubations carried out in 0.1M TEA-HCl, 2mM $MgCl_2$, pH 8.0, 26°C.

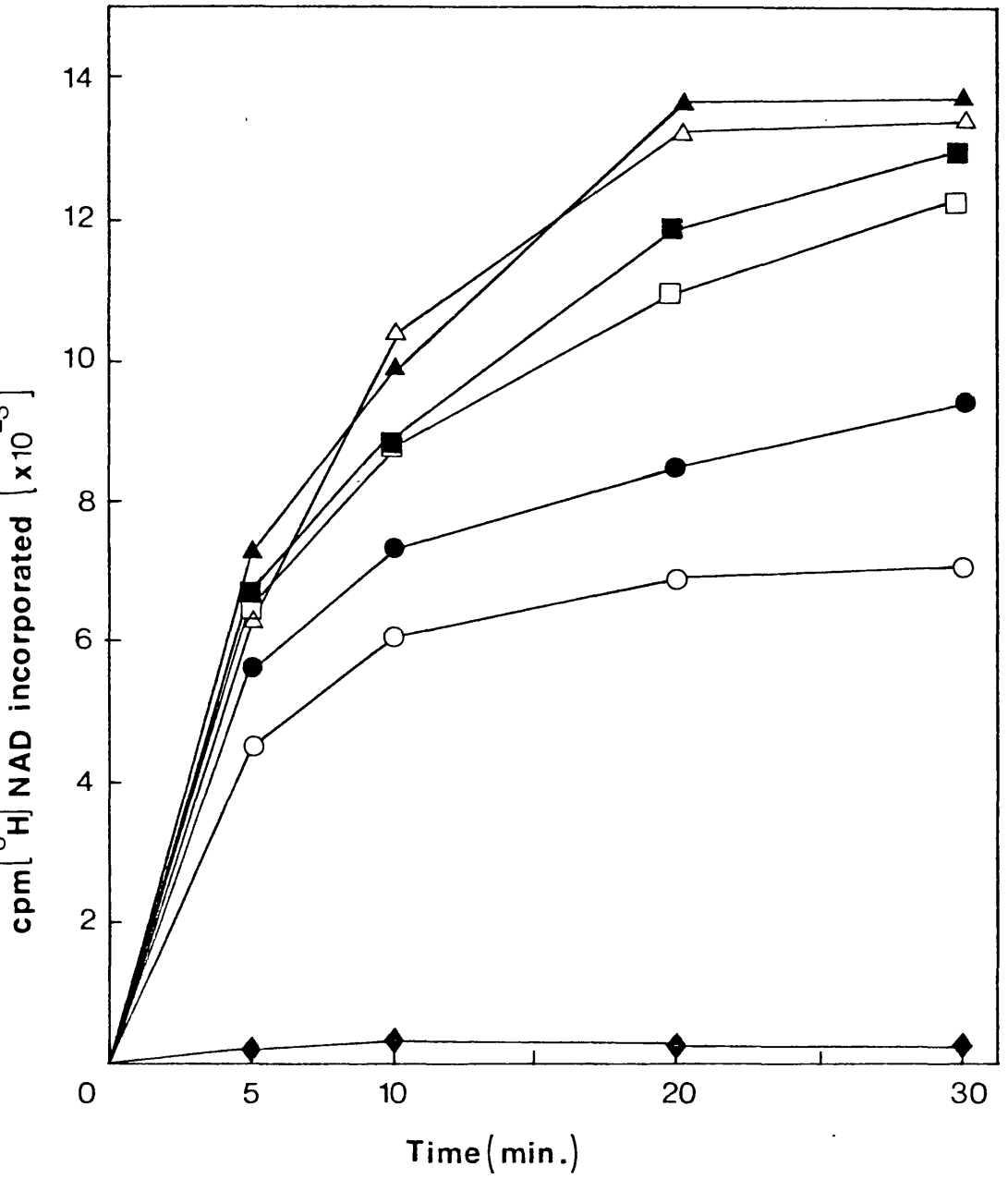

14
12
10
8
6
4
2
0
5
10
20
30
cpm [3H] NAD incorporated [x10-3]
Time (min.)

TABLE 4.1.A. Effect of $Hg(CH_3CO_2)_2$ on $[^3H]$ NAD incorporation by a nuclear preparation isolated from wheat embryos

Treatment	% acid insoluble cpm *
Control	100.0
100 mM Nicotinamide	2.2
0.5 mM $Hg(CH_3CO_2)_2$	1.7
1.0 " "	2.1
2.5 " "	2.5
5.0 " "	2.6
7.5 " "	2.3
10.0 " "	2.4

TABLE 4.1.B. Effect of β mercaptoethanol upon the inhibitory effect of Hg^{2+}

Treatment	% acid insoluble cpm **
Control	41.8
+ 20 mM β mercaptoethanol	100.0
+ 1 mM $Hg(CH_3CO_2)_2$	0.7
+ 1 mM $Hg(CH_3CO_2)_2$ + 20 mM β mercaptoethanol	93.9

all assays carried out at 26^o in 0.1M TEA-HCl, 2mM $MgCl_2$ pH8.

* 100% = 11250 cpm
** 100% = 35925 cpm

Fig. 4.6. Effect of Mg^{2+} on poly (ADP-Ribose) polymerase activity in isolated wheat nuclei

○ = control (no $MgCl_2$)

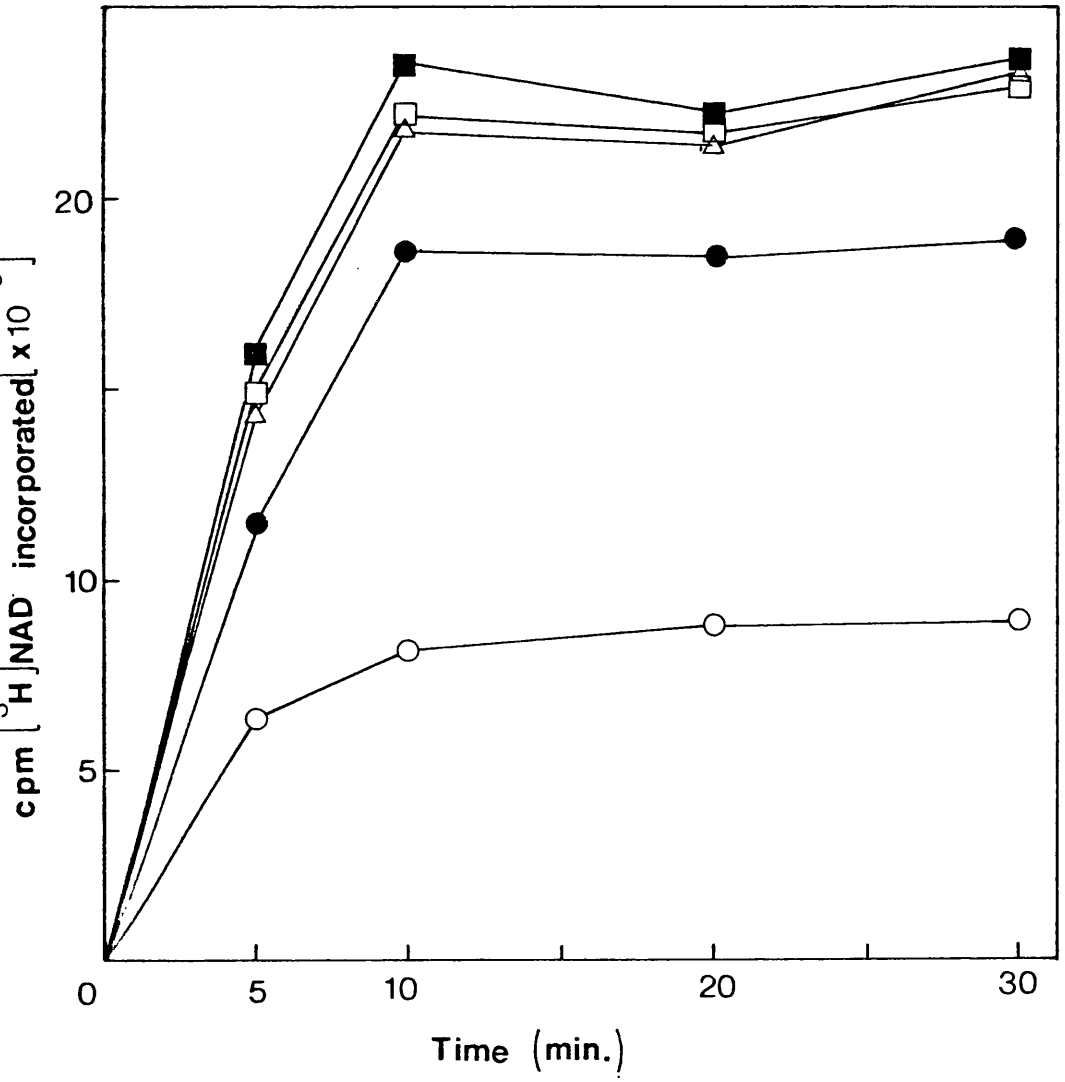
● = 1mM $MgCl_2$

□ = 2mM "

■ = 5mM "

△ = 10mM "

Incubations carried out in 0.1M TEA-HCl, 10mM β mercaptoethanol, pH 8.0, 26°C.


cpm [^{3}H]NAD incorporated [x10^{-3}]
20
10
5
0
5
10
20
30
Time (min.)

4.9. Effect of Salts

The effect of $(NH_4)_2SO_4$, NH_4Cl, Na_2SO_4 and NaCl on the wheat poly (ADP-Ribose) polymerase was investigated as described below. The standard assay conditions were modified by dissolving crystalline salt in the nuclear preparation, to give final concentrations of 0.25, 0.5, 1.0, 1.5, 2.0 and 2.5 M of the respective salts. However, it proved impossible to dissolve Na_2SO_4 above 1.5M. In addition to the normal control (no salt) an enzyme blank was used where the polymerase was completely inhibited by 100 mM nicotinamide. This nicotinamide blank was used to ensure that no non-specific binding of [^{3}H] NAD occurred. The pH of the various reaction mixtures was also checked to ensure that no pH change of the reaction mixture occurred upon the addition of the various salts. No significant pH change was seen. The results, expressed as a percentage activity (after subtraction of the nicotinamide control) are shown in Fig. 4.7.

4.10. Effect of Nucleotides and Polynucleotides

Since a variety of nucleotides and polynucleotides are known to affect poly (ADP-Ribose) polymerase in other systems (Hilz and Stone 1976), an investigation into the effects of a variety of such compounds on the wheat system was made. The compounds used were AMP, cylic AMP, ATP, GMP, GTP, ADP-Ribose, single and double strand DNA and RNA. The standard assay conditions were employed with the addition of the various compounds. The single stranded DNA was prepared by heating a solution of native calf thymus DNA in a water bath at 100^{o}C for 10 minutes, then quenching on ice. As can be seen(Table 4.2.), AMP, cyclic AMP, ATP,

Fig. 4.7. Effect of salts on poly (ADP-Ribose) polymerase activity in isolated wheat nuclei

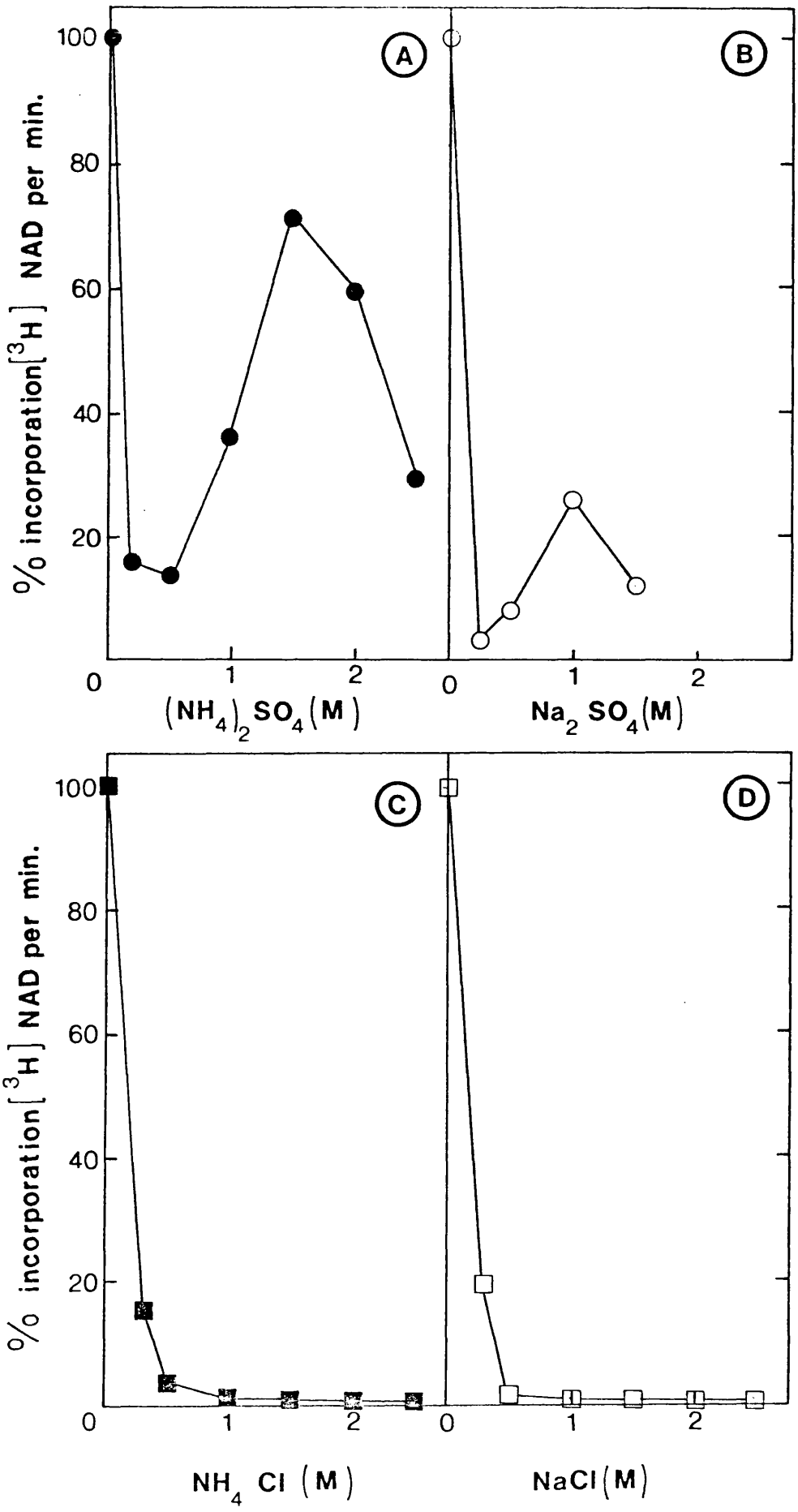
A = $(NH_4)_2\ SO_4$

B = $Na_2\ SO_4$

C = $NH_4\ Cl$

D = Na Cl

Incubations carried out in 0.1M TEA-HCl, 2mM $MgCl_2$, 10mM β mercaptoethanol, pH 8.0, 26°C.


A
B
C
D
100
80
60
40
20
0
1
2
% incorporation [3H] NAD per min.
(NH4)2 SO4 (M)
Na2 SO4 (M)
NH4 Cl (M)
NaCl (M)

GMP, GTP and ADP-Ribose are all inhibitory. Of these compounds the most effective inhibitors were ATP and GTP.

4.11. Inhibition Studies

Two inhibitors have been employed in this study, the first, nicotinamide, is widely recognised as an inhibitor of poly (ADP-Ribose) polymerase (Hilz and Stone 1976). The second, 3-amino-benzamide, has been discovered to be a potent inhibitor of poly (ADP-Ribose) polymerase by workers in this laboratory (Purnell and Whish 1980). When nicotinamide was present in the standard assay (above) a dose-dependent inhibition of $[^3H]$ NAD incorporation was observed. When an NAD concentration of 100 μM was employed, 50% inhibition was obtained at a concentration of 115 μM nicotinamide, (Fig. 4.8.). Under the normal standard assay conditions (2.5 uM NAD) 100% inhibition was seen at 150 μM nicotinamide and 50% inhibition at 60 μM nicotinamide. When 3-amino benzamide (3AB) was employed in the standard assay inhibition of the poly (ADP-Ribose) polymerase was also seen. This gave 100% inhibition at 100 μM 3AB and 50% inhibition at 9 μM 3AB (Fig. 4.9.). A more detailed study of the inhibition kinetics is given below in Section 4.12.

4.12. Kinetic Analyses

A K_m determination for the wheat poly (ADP-Ribose) polymerase was performed using the standard assay procedure, initially using NAD concentrations in the range 100 μM - 500 μM. The $[^3H]$ NAD incubation was stopped after 2 minutes because at these higher NAD concentrations the linearity of the incorporation occurs for a much shorter period of time i.e.

TABLE 4.2. Effect of various compounds on poly (ADP-Ribose) polymerase activity in isolated wheat nuclei

Treatment	% incorporation *
Control	100.0
1mM AMP	95.0
10mM AMP	94.3
1mM cyclic AMP	69.3
10mM cyclic AMP	77.3
1mM ATP	65.3
10mM ATP	27.3
1mM GMP	99.5
10mM GMP	41.2
1mM GTP	71.8
10mM GTP	20.5
1mM ADPR	106.3
10mM ADPR	89.2
10 µg.ml. ss DNA	100.8
10 µg.ml. ds DNA	95.3
10 µg.ml. RNA	101.7

* 100% incorporation = 18,600 cpm per 10 µl aliquot

Fig. 4.8. Effect of nicotinamide on poly (ADP-Ribose) polymerase activity in isolated wheat nuclei

Incubations carried out in 0.1M TEA-HCl, 2mM $MgCl_2$, 10mM β mercaptoethanol, pH 8.0, 26°C. [NAD] = 100 μM.

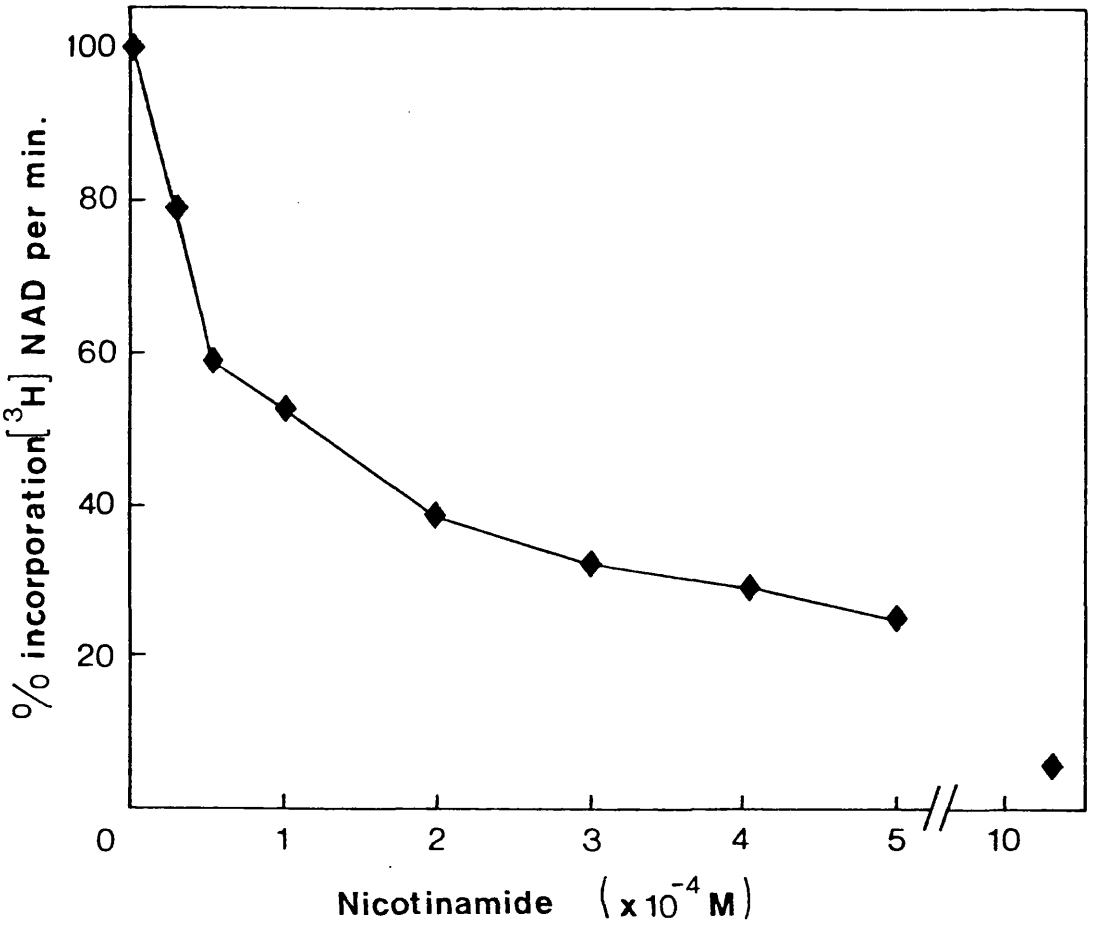
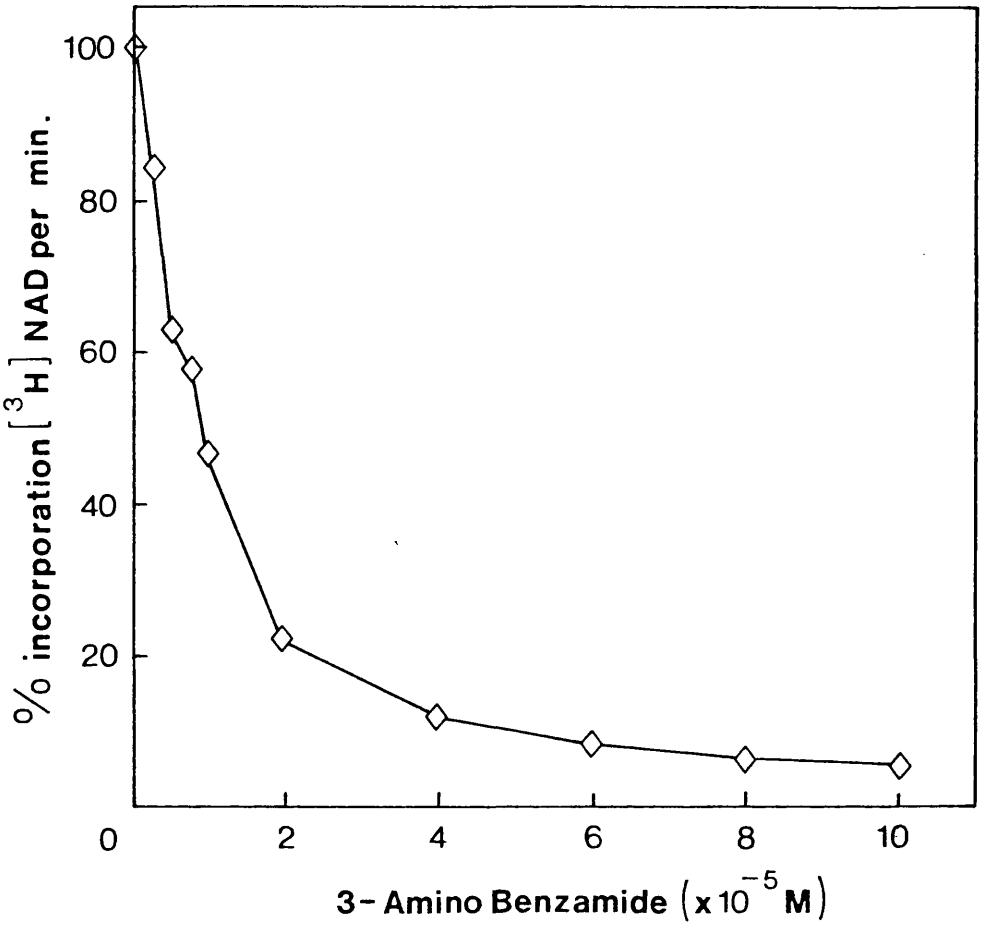

100
80
60
40
20
0
1
2
3
4
5
10
% incorporation [3H] NAD per min.
Nicotinamide (x 10-4 M)

Fig. 4.9. Effect of 3-aminobenzamide on poly (ADP-Ribose) polymerase activity in isolated wheat nuclei

Incubations carried out in 0.1M TEA-HCl, 2mM $MgCl_2$, 10mM β mercaptoethanol, pH 8.0, 26°C. [NAD] = 10 μM.


100
80
60
40
20
0
2
4
6
8
10
% incorporation [3H] NAD per min.
3- Amino Benzamide (x 10-5 M)

2 minutes. A velocity (V) against substrate (S) plot is shown in Fig. 4.10. The K_m was estimated by two methods. The first was using the Lineweaver-Burk double reciprocal plot which gave a K_m of $7.4 \pm 1.2 \times 10^{-5}$ M (Fig. 4.11.). Secondly the Eisenthal direct linear plot (Eisenthal and Cornish-Bowden, 1974) was used and gave a K_m value of $7.5 \pm 1.4 \times 10^{-5}$ M (Fig. 4.12.). Further kinetic studies using inhibitors of poly (ADP-Ribose) polymerase (4.11. above) revealed that at low NAD concentrations (20 - 100 uM) a second K_m could be derived from the Lineweaver-Burk plot, the calculated value of this second K_m was 5.7×10^{-6} M (Fig. 4.13.). The implication of these observations is discussed below.

The inhibition of the wheat poly (ADP-Ribose) polymerase by nicotinamide and 3AB (above) was subjected to kinetic analysis in order to determine the nature of the inhibition. The procedure followed was exactly as given above, except that the range of $[^3H]$ NAD concentrations used was 20 - 300 μM and the nuclear preparation had been stored at -20^oC overnight. This pretreatment of the nuclear preparation results in total loss of poly (ADP-Ribose) glycohydrolase activity (personal communication, N. Abed). The results obtained are shown in Fig. 4.13. and 4.14. for nicotinamide and 3AB respectively.

4.13. Concluding Remarks

The conclusions which can be drawn from the results presented above are as follows:-

(a) Poly (ADP-Ribose) is synthesised by preparations of isolated wheat nuclei *in vitro*. This poly (ADP-Ribose) has an average chain length of 2.51 ± 0.09. This value closely compares with

FIG. 4.10. Effect of increasing NAD concentrations upon poly (ADP-Ribose) polymerase in isolated wheat nuclei

Incubations carried out in 0.1M TEA HCl, 2mM $MgCl_2$, 10mM β mercaptoethanol, pH 8.0, 26°C

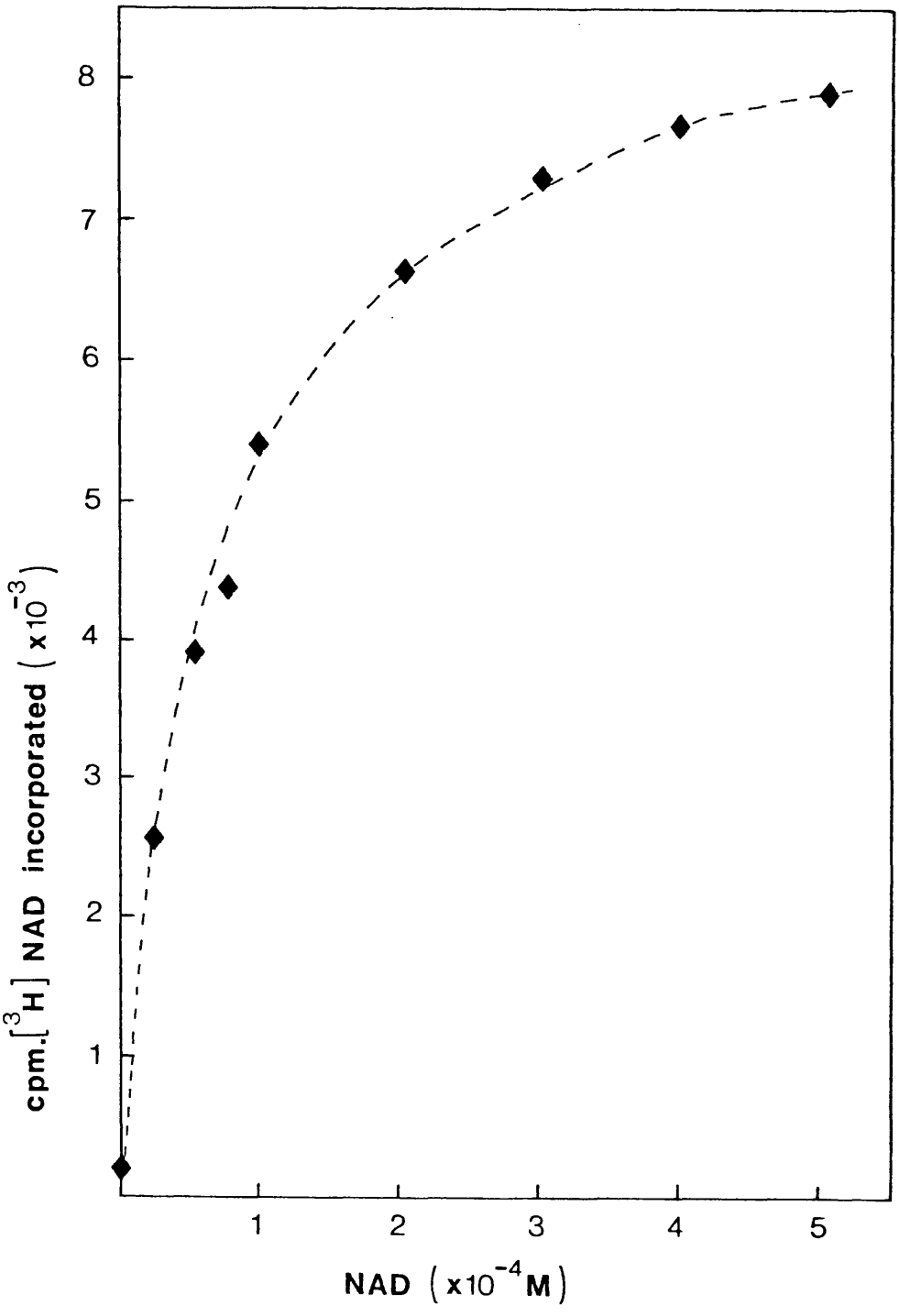

8
7
6
5
4
3
2
1
cpm.[3H] NAD incorporated (x10-3)
1
2
3
4
5
NAD (x10-4M)

FIG. 4.11. Effect of NAD concentration on poly (ADP-Ribose) polymerase in isolated wheat nuclei:-

Lineweaver-Burk plot

Incubations carried out in 0.1M TEA HCl, 2mM $MgCl_2$, 10mM β mercaptoethanol, pH 8.0, 26°C.

V = cpm incorporated per 2 minutes

S = NAD (μM)

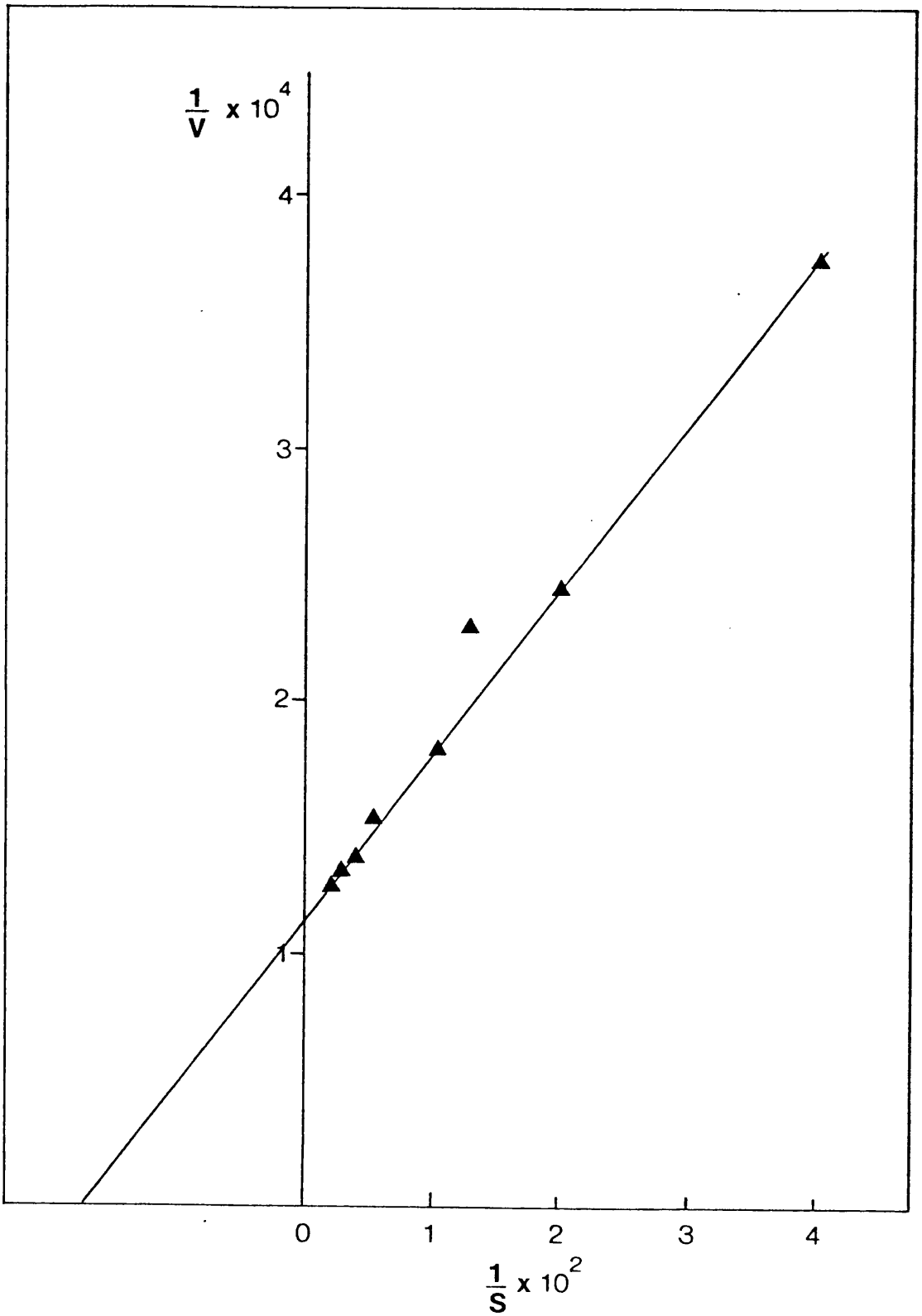

1/V x 10^4
4
3
2
1
0
1
2
3
4
1/S x 10^2

FIG. 4.12. Effect of NAD concentration on poly (ADP-Ribose) polymerase in isolated wheat nuclei:- Eisenthal-Cornish-Bowden plot

Incubations carried out in 0.1M TEA HCl, 2mM $MgCl_2$, 10mM β mercaptoethanol, pH 8.0, 26°C.

V = cpm incorporated per 2 minutes

S = NAD(μM)

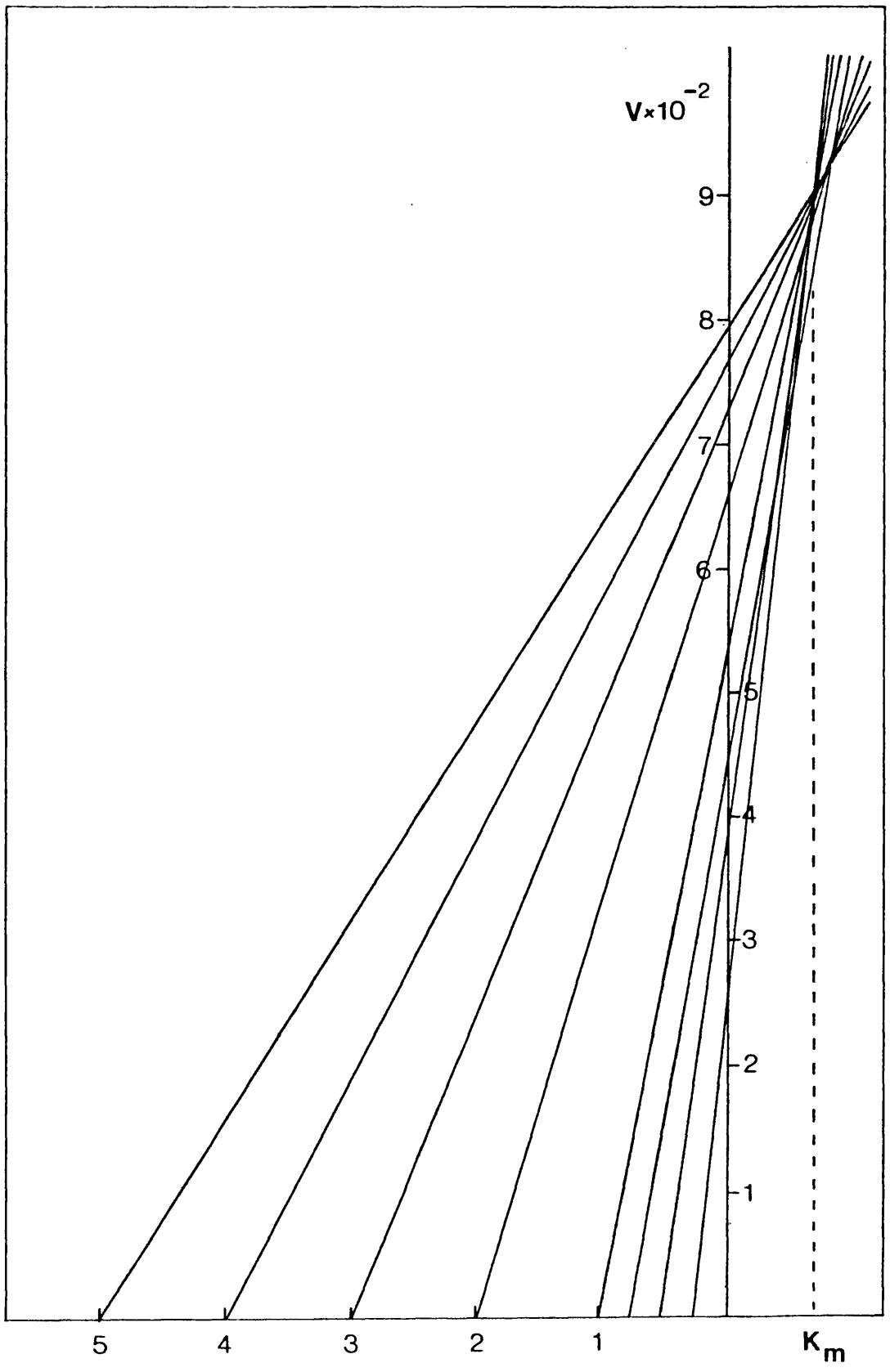

V×10^-2
9
8
7
6
5
4
3
2
1
5
4
3
2
1
Km
-[S] × 10^-2

FIG. 4.13. Inhibition of poly (ADP-Ribose) polymerase in isolated wheat nuclei by nicotinamide:-

Lineweaver-Burk plot

Incubations carried out in 0.1M TEA-HCl, 2mM $MgCl_2$, 10mM β mercaptoethanol, pH 8.0, 26°C.

V = cpm incorporated per 2 minutes

S = NAD (μM)

O = control (no nicotinamide)

◇ = 50 μM nicotinamide

▲ = 100 μM "

△ = 200 μM "

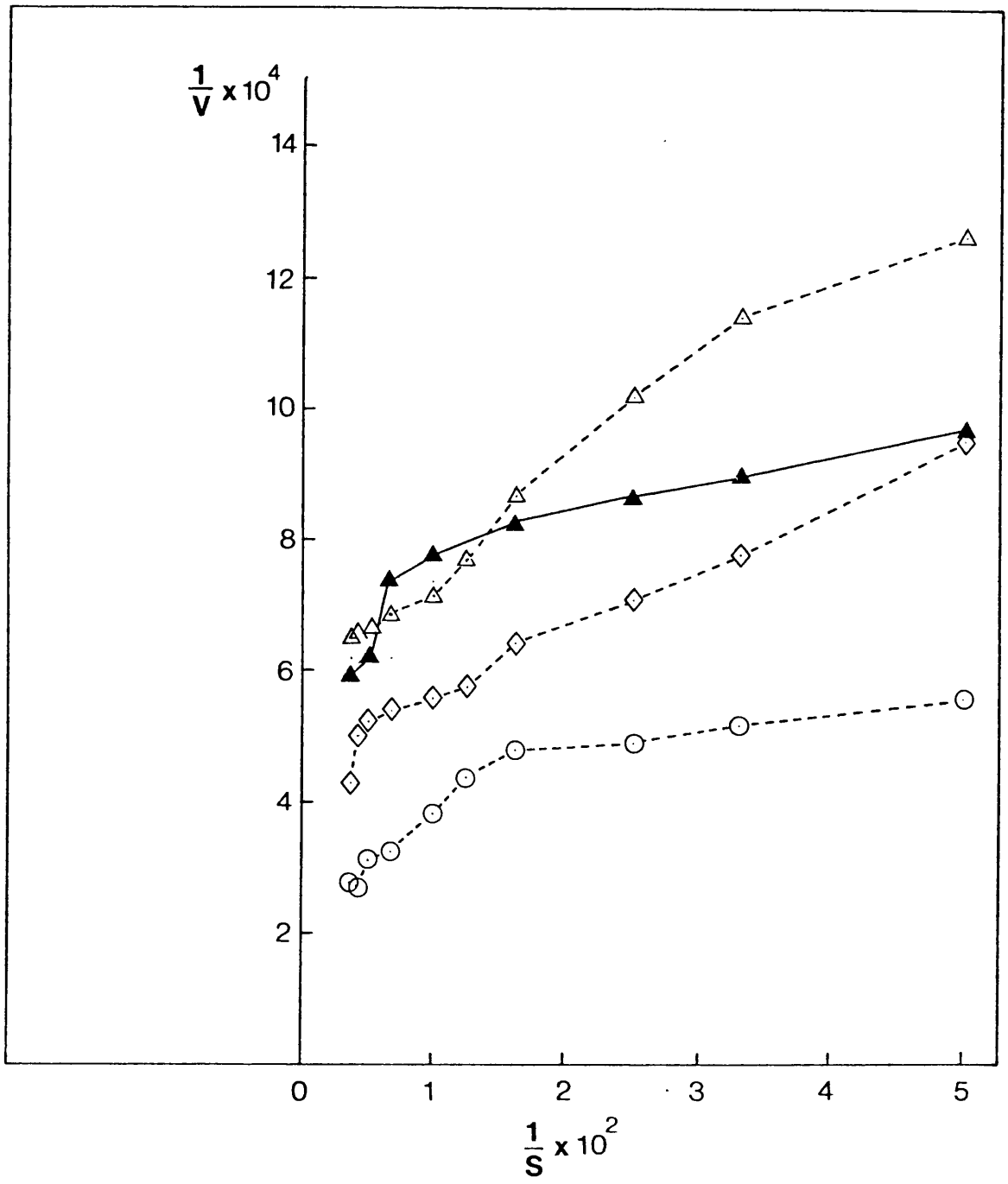

1/V x 10^4
14
12
10
8
6
4
2
0
1
2
3
4
5
1/S x 10^2

FIG. 4.14. Inhibition of poly (ADP-Ribose) polymerase in isolated wheat nuclei by 3-amino benzamide:- Lineweaver-Burk plot

Incubation carried out in 0.1M TEA HCl, 2mM $MgCl_2$, 10mM β mercaptoethano, pH 8.0, 26°C.

V = cpm incorporated per 2 minutes

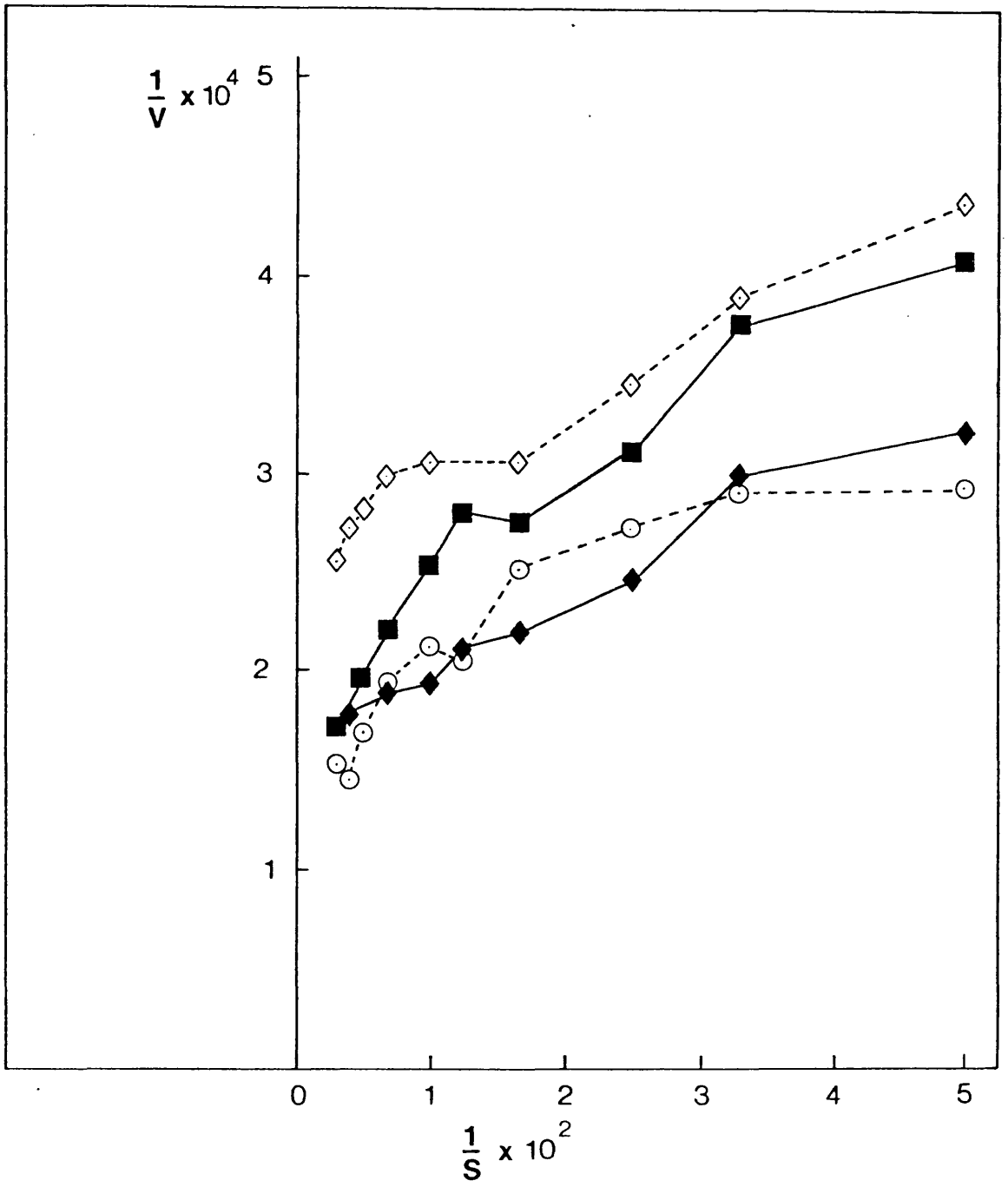
S = NAD (μM)

0 = control (no 3-amino benzamide)

◆ = 50 μM 3-amino benzamide

■ = 100 μM " "

◇ = 200 μM " "


1/V x 10^4
5
4
3
2
1
0
1
2
3
4
5
1/S x 10^2

a reported range of 1.2 to 2.9 in rat liver (Reeder et al, 1967, Nishizuka et al, 1968, Shima et al, 1970, Hilz et al, 1972), 1-4 in mouse LS cells (Stone, 1973) and 5 in mouse lymphoma (Lehman et al, 1974). Other animal tissues have been examined, full details can be found in Hilz and Stone (1976). The slime mould Physarum polycephalum, which in some respects may be considered to have plant-like characteristics,has been shown to synthesise poly (ADP-Ribose) with an average chain length of 6 (Brightwell et al, 1975).

(b) The observed temperature optimum of 17°C is in agreement with the observation noted by Hilz and Stone (1976) that the optimal temperature for in vitro synthesis of poly (ADP-Ribose) is 10°-15°C below the growth temperature of the cells from which the nuclei are derived. The optimal growth conditions for wheat seeds include a requirement for temperatures in the range 25°-35°C.

(c) The observed pH optimum for NAD incorporation of pH 7.9 agrees well with reports for other systems. An optimum of pH 7.8 in rat pancreas (Poirer et al, 1978), pH 8 in BHK cells and rat liver nuclei (Furneaux and Pearson, 1977, Fujimura et al, 1967), pH 8.2 in Physarum nuclei (Brightwell and Shall, 1971) and pH 8.5 in mouse LS cells (Stone and Shall, 1973).

(d) The wheat poly (ADP-Ribose) polymerase requires -SH groups for optimal activity. Again, all other reports of poly (ADP-Ribose) polymerase in other systems indicate a similar requirement for free - SH groups. Where the necessary concentrations have been reported the values given are in close agreement to those obtained in this study. In the wheat system the optimum ß mercaptoethanol concentration is 10 mM, Yamada et al (1971)

have reported an optimum of 2 mM for both β mercaptoethanol and DTT, using isolated rat liver nuclei. Stone and Shall (1973) have shown that in isolated LS cell nuclei both β mercaptoethanol and DTT enhanced incorporation up to an optimum of 10 mM. What does appear to be unusual about the wheat system is that the inhibition by Hg^{2+} is reversible. No published data is available for other systems and work in this laboratory using isolated pig thymus nuclei and identical experimental conditions gave only a 50% recovery of activity (personal communication, W.J.D. Whish).

(e) The wheat system is again like all other reported systems in that there is a Mg^{2+} requirement to give maximal activity. (Chambon et al, 1966, Nishizuka et al, 1967, Yamada et al, 1971, Shall et al, 1972, Stone and Shall 1973, Furneaux and Pearson, 1977, Poirer et al, 1978). The 2mM Mg^{2+} optimum seen in the wheat system is in close agreement with the other reports which gave a range of 2 - 20 mM Mg^{2+}. Although not examined in this study, the suggestion by Stone and Shall (1973) that the NAD concentration used in the assay influenced the optimum Mg^{2+} concentration requirement, could account for the reported differences. That is, an NAD - Mg^{2+} complex is the enzyme substrate rather than NAD alone.

(f) The effect of $(NH_4)_2\ SO_4$ on the wheat poly (ADP-Ribose) polymerase (see Fig. 4.7.) is very similar to that reported by Ueda et al (1968) using isolated rat liver nuclei. Ueda et al showed the same pattern of activity, with a peak (40% of control value) at 1.7 M $(NH_4)_2\ SO_4$. The present study has shown a peak of activity at 1.5 M $(NH_4)_2\ SO_4$. The incorporation seen at this salt concentration was 70% of the control

(no salt) value. Stone and Shall (1973) failed to demonstrate any such effect using isolated LS cell nuclei. The results given in the following chapter (5.10.) clearly show that the activity at 1.5 M $(NH_4)_2$ SO_4 was not due to fluctuations in the degradation of poly (ADP-Ribose) with changing salt concentrations (Fig. 5.5.) as has been postulated by Ueda et al. When the effects of the various salts used were compared both NaCl and NH_4Cl were directly comparable, giving 100% inhibition of the polymerase at high concentrations. However, when Na_2SO_4 was used a similar, but less marked, effect to that obtained with $(NH_4)_2$ SO_4 was seen. In the case of Na_2SO_4 the activity maximum occurred at 1.0M, but was only 25% of the control (no salt) incorporation. These results suggest that the observed effect was due, at least in part, to the presence of SO_4^{2-}. It would therefore seem quite possible that the observed activity in high salt was due to ionic effect on the polymerase, possibly by making the polymerase more accessible to NAD. Other possibilities include (a) dissociation of chromatin, (b) the histones are made more available for ADP-Ribosylation, and (c) allowing the polymerase to bind to naked DNA, thus stimulating activity (see 1.2.).

(g) Of the various nucleotides examined only cyclic AMP (cAMP), ATP, GMP and GTP had any significant inhibitory effect on the wheat polymerase (see Table 4.3.). One possible explanation for the effect mediated by ATP was that the nuclear preparation contained significant NAD pyrophosphorylase activity and NMN. This would result in the production of 'cold' NAD resulting in a lower specific activity of the $[^3H]$ NAD in the assay, which in turn will result in an apparent inhibition of the polymerase.

However, it is more difficult to explain the effect of cAMP, GMP and GTP. It may be that GMP is converted to GTP by the nuclear preparation, but this supposes that GTP can be converted to an NAD analogue and that the analogue successfully competes with the [^{3}H] NAD in the formation of poly (ADP-Ribose). Another, more likely, suggestion is that a general nucleotide-phosphate inhibition is occurring, this proposal would fit all the compounds noted above. The inhibition by high concentrations of ADP-Ribose could well be a form of product inhibition. Unfortunately no unlabelled poly (ADP-Ribose) was available to test this hypothesis. The lack of effect by the nucleicacids used may be due to the fact that the enzyme used was not highly purified. Previously DNA has only been shown to have an effect using highly purified preparations (see Hilz and Stone 1976).

(h) At high NAD concentrations (greater than 100 μm) the wheat poly (ADP-Ribose) polymerase has a K_m of 7.5×10^{-5} M. This value is in good agreement with reports of other systems e.g. 4×10^{-4} M in quail oviduct (Muller and Zahn, 1975); 2.5×10^{-4} M in rat pancreas (Poirer et al, 1978); 1.5×10^{-3} M in LS cell nuclei (Stone and Shall, 1973). However, as noted above (4.11.) a second K_m was apparent at low NAD concentrations (20-100 μM). These two K_m values may have been an artefact due to a limiting Mg^{2+} concentration, in line with results reported by Stone and Shall (1973). These workers noted that the optimum Mg^{2+} concentration increased with increasing NAD concentrations in the assay conditions employed. To test this hypothesis the K_m determination at high NAD concentrations was repeated, but at an Mg^{2+} concentration of 20 mM. This experiment gave no significant variation in the K_m obtained. It was

therefore concluded that there might be two poly (ADP-Ribose) polymerase activities in isolated wheat nuclei. Ueda and others (1.2.) have postulated at least two poly (ADP-Ribose) polymerase activities and Dietrich and Siebert (1973) have demonstrated the presence of two enzymes in rat liver nuclei. They found two species of poly (ADP-Ribose), one hydroxylamine sensitive and one hydroxylamine insensitive. The K_m for the formation of these was 8.7×10^{-5} M and 6.5×10^{-4} M respectively. They also noted that at low NAD concentrations more hydroxylamine sensitive ADP-Ribosylation occurred.

The observation that nicotinamide gives 50% inhibition of poly (ADP-Ribose) polymerase activity, at approximately equimolar concentrations, is in agreement with other workers (Hilz and Stone, 1976). However, when the inhibition kinetics are examined in detail, the wheat system does appear to be different from other systems. Reports have shown nicotinamide to act as a competitive inhibitor but this does not appear to be the case in the wheat system. At low (up to 100 μM) nicotinamide concentrations and high NAD concentrations the inhibition appears to be competitive, at low NAD concentrations the inhibition seems to be uncompetitive. Furthermore, at high nicotinamide concentrations (200 μM) the inhibition appears to be uncompetitive. A very similar picture was seen with 3AB. Repeated attempts to resolve the inhibition kinetics failed to clarify the situation. It was therefore concluded that the inhibition of the wheat poly (ADP-Ribose) polymerase does not follow simple Michaelis-Menten kinetics.

5. POLY (ADP-RIBOSE) GLYCOHYDROLASE

5.1. Preliminary Investigations

Once the presence of poly (ADP-Ribose) polymerase activity had been demonstrated in wheat embryos, the nuclear preparations were examined for their ability to hydrolyse poly (ADP-Ribose)

Preliminary experiments were performed using the standard assay conditions (4.2.), incubating for 10 minutes then making the preparation 200 μM with respect to nicotinimide or 150 μM with respect to 3AB (4.11.). Aliquots were removed at intervals and counted for acid insoluble radioactivity. The initial results showed that nuclear preparations from wheat embryos were able to hydrolyse $[^3H]$ poly (ADP-Ribose) to acid soluble material. The degradation of poly (ADP-Ribose) could have been due to one or more of several possibilities:-

(a) chemical hydrolysis of poly (ADP-Ribose) or the poly (ADP-Ribose) -protein bond.

(b) phosphodiesterase activity. This would have given rise to the formation of 2' - (5" - phosphoribosyl) 5'-AMP (PRAMP) and 5' AMP via the cleavage of the pyrophosphate bonds of poly (ADP-Ribose). (Fig. 1.2.b.).

(c) poly (ADP-Ribose)glycohydrolase activity, yielding ADP-Ribose following cleavage of the glycosidic bonds.

In case (b) the PRAMP could be further hydrolysed to ribosyl adenosine (by alkaline phosphatase), whereas in case (c) treatment with alkaline phosphatase would lead to adenosine and ribose-5-phosphate.

5.2. Standard Assays

Two standard assays were developed. The first assay involved incubating isolated nuclei in 0.1M TEA-HCL, 0.2mM

$MgCl_2$, 10mM β mercaptoethanol, pH 8, sonicating, then incubating with 2.5 μM [^{3}H] NAD at 26^{o}C for 10 minutes. The samples were then made 150 μM with respect to 3AB and incubated at 26^{o}C. Aliquots were removed at intervals to determine acid insoluble radioactivity. The second assay used the exogenous addition of [^{3}H] poly (ADP-Ribose) prepared as described in 2.9b. The isolated nuclei were prepared as for the endogenous assay, the difference being that the incubation with [^{3}H] NAD, followed by the addition of 3AB was omitted. Instead approximately 4,000 c.p.m. per 10 μl of [^{3}H] poly (ADP-Ribose)was added. The latter assay was used unless stated otherwise.

To test whether chemical hydrolysis (case 5.1a. above) was occurring two control experiments were designed:-

(a) [^{3}H] poly (ADP-Ribose) was incubated with 0.1M TEA-HCL, 2mM $MgCl_2$, 10mM β mercaptoethanol, pH 8.0 at 26^{o}C for a period of 8 hours, removing aliquots at intervals and counting acid insoluble radioactivity. Over this time there was no significant loss of acid insoluble radioactivity.

(b) A nuclear preparation was incubated with [^{3}H] NAD as standard (4.2.) and then ethanol precipitated. The precipitate was then redissolved in 0.1M TEA-HCL, 2mM $MgCl_2$, 10mM β mercaptoethanol, pH 8.0, and incubated as described for 5.2a. (above). Again no significant loss of acid insoluble radioactivity was seen. It was therefore concluded that the hydrolysis of [^{3}H] poly·(ADP-Ribose) by isolated wheat nuclei was due to an activity in the nuclear preparation.

5.3. Effect of Fluoride

In order to check that there was no phosphodiesterase

activity isolated nuclei were incubated using the standard assay, but with varying concentrations of NaF. The results are shown in Table 5.1. and clearly show that fluoride has no effect on the hydrolysis of poly (ADP-Ribose) by isolated wheat nuclei. As fluoride is known to be a phosphodiesterase inhibitor (Bernardi and Bernardi, 1971) these results showed that the hydrolysis is not due to phosphodiesterase-like activity.

5.4. T.L.C. Analysis of breakdown of [^{3}H] poly (ADP-Ribose)

In order to resolve 5.1b. and 5.1c. (above) the reaction products of the standard assay were examined using t.l.c. analysis. Using t.l.c. system II (2.11.) it is apparent that isolated wheat nuclei metabolise [^{3}H] poly (ADP-Ribose) to a greater extent than other workers have found. Fig. 5.1a. shows the reaction products after 1 hour incubation, Fig. 5.1b. shows the same reaction mixture after 2 hours incubation. These results do not conclusively support the idea of either phosphodiesterase or glycohydrolase activity. As a result t.l.c. system III was used to separate adenosine and ribosyl adenosine. As no authentic ribosyl adenosine was available some [^{3}H] labelled ribosyl adenosine was prepared. This was done by incubating [^{3}H] poly (ADP-Ribose) (2.9b.) with SVPDE followed by incubation with alkaline phosphatase (full conditions given in 2.13.). Descending paper chromatography (2.12.) was tried but failed to separate adenosine and ribosyl adenosine. When the reaction products were examined using t.l.c. system III, a single band was observed (Fig. 5.2a.), which shows that system III can separate adenosine and ribosyl adenosine.

TABLE 5.1. Effect of NaF upon the hydrolysis of [^{3}H] poly (ADP-Ribose) by a nuclear preparation isolated from wheat embryos

Concentration of NaF (mM)	% acid insoluble cpm lost * per 10 minutes
0	10.73
1	11.23
2	10.11
5	12.55
10	11.51

* 100% cpm = approx, 13220

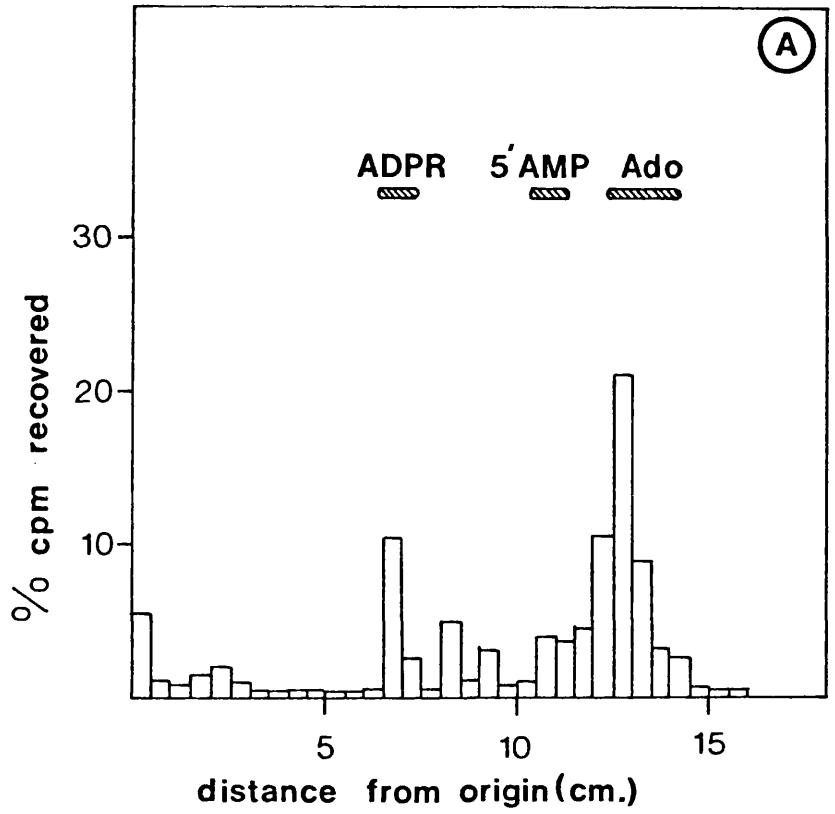
assay performed at 26^{o}C in 0.1M TEA-HCl, 2mM $MgCl_2$, 10mM β mercaptoethanol pH8.

Fig. 5.1. Representative t.l.c. analysis of the hydrolysis of $[^3H]$ poly (ADP-Ribose) by isolated wheat nuclei

A = after incubation at 26°C for 1 hour

B = " " " " " 2 hours

t.l.c. system II was used, markers employed were ADPR, 5' AMP and Ado.


A
ADPR
5'AMP
Ado
30
20
10
% cpm recovered
5
10
15
distance from origin (cm.)

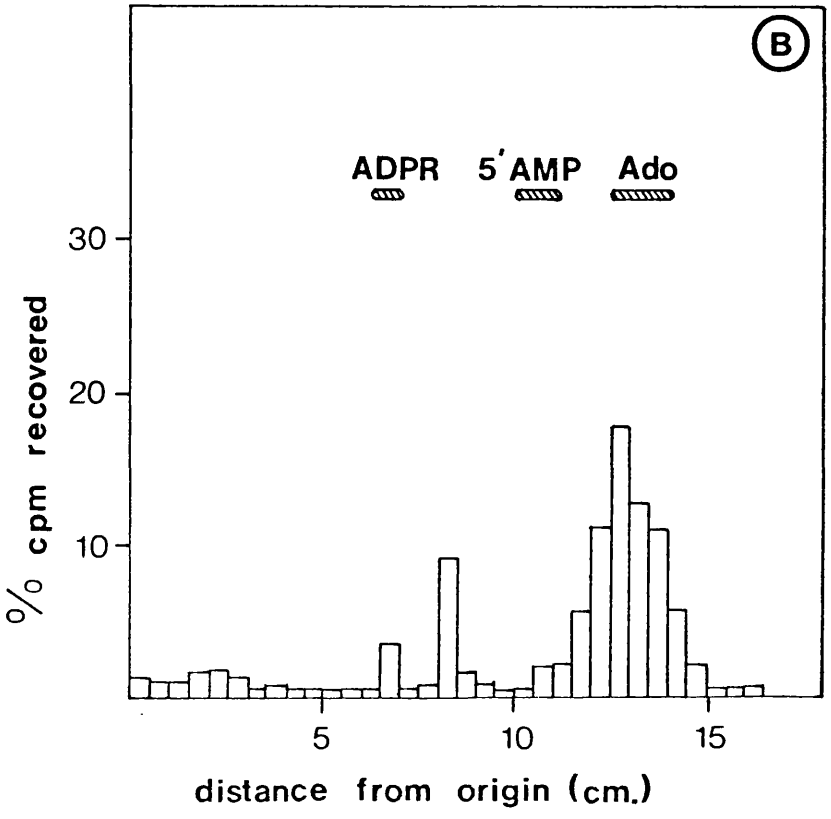
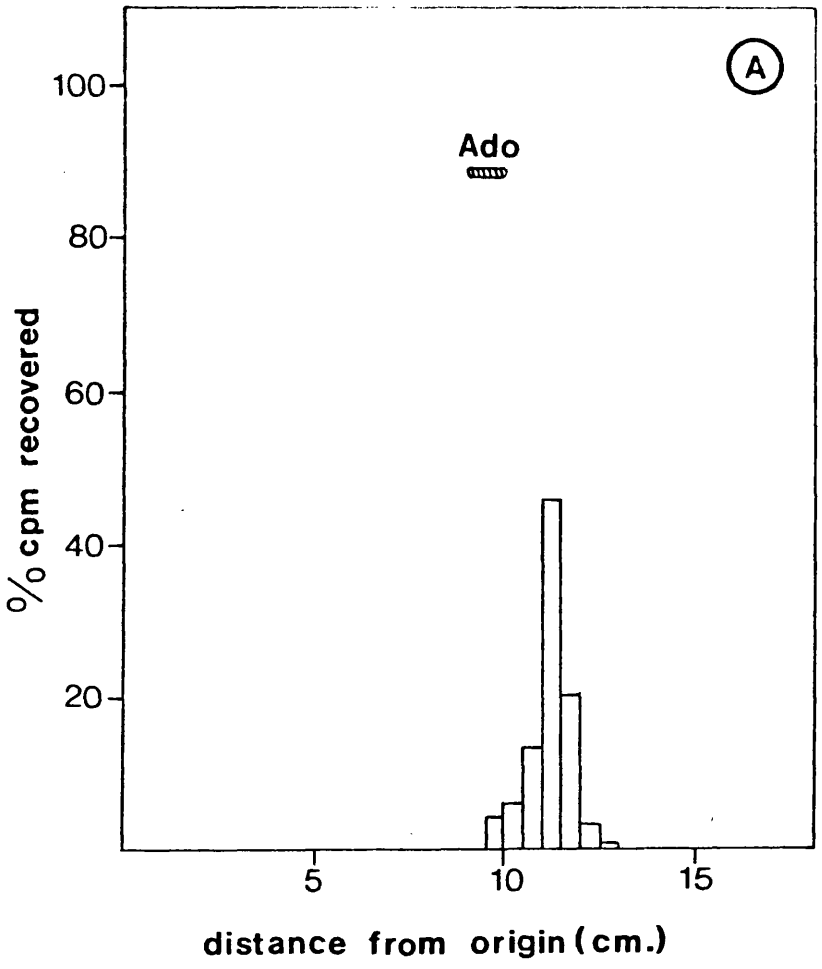

B
ADPR
5'AMP
Ado
30
20
10
% cpm recovered
5
10
15
distance from origin (cm.)

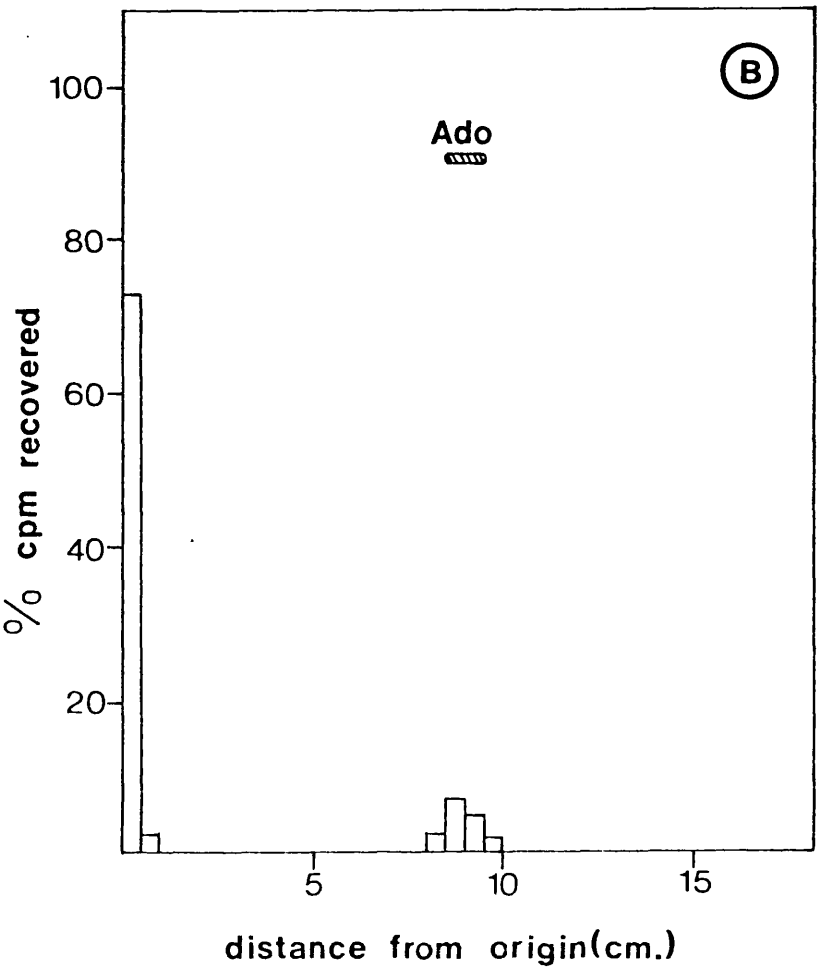
Fig. 5.2.A. Representative t.l.c. of the digestion of $[^3H]$ poly (ADP-Ribose) by SVPDE followed by Alkaline Phosphatase

Solvent system III was used, the marker employed was Adenosine (Ado)

Fig. 5.2.B. Representative t.l.c. of reaction mixture obtained by incubating $[^3H]$ poly (ADP-Ribose) with isolated wheat nuclei, as shown in Fig. 5.1.

Solvent system III was used, the marker employed was Adenosine (Ado)


A
Ado
100
80
60
40
20
% cpm recovered
5
10
15
distance from origin (cm.)


B
Ado
100
80
60
40
20
% cpm recovered
5
10
15
distance from origin(cm.)

The reaction products of the standard assay were subjected to digestion by SVPDE and alkaline phosphatase, followed by t.l.c. analysis using system III. As can be seen (Fig. 5.2b) no [^{3}H] ribosyl adenosine was detected, showing that phosphodiesterase activity is not responsible for the degradation of [^{3}H] poly ADP-Ribose.

5.5. Poly (ADP-Ribose) Glycohydrolase in Wheat

It was concluded that isolated wheat nuclei contain poly (ADP-Ribose) glycohydrolase and that most if not all *in vitro* degradation of poly (ADP-Ribose) occurs via the glycohydrolase with subsequent degradation of the ADP-Ribose. Phosphodiesterase activity cannot be completely ruled out as it is not known if PRAMP can be rapidly hydrolysed in isolated wheat nuclei to ribosyl adenosine. However, when [^{3}H] PRAMP, prepared from [^{3}H] poly (ADP-Ribose), is incubated with isolated wheat nuclei it is only slowly converted to ribosyl adenosine. The extensive degradation of ADP-Ribose by isolated wheat nuclei is in good agreement with the work of Wu *et al* (1978). These workers have shown, using rabbit reticulocyte lysates, that ADP-Ribose, derived from NAD via NAD glycohydrolase, a ubiquitous cytoplasmic enzyme, is rapidly converted to ATP via AMP and ADP *in vitro*.

5.6. Temperature Dependance

The temperature optimum of the glycohydrolase was determined by using the standard incubation procedure. Aliquots were removed at 5 minute intervals and acid insoluble radioactivity determinations carried out. The rate of hydrolysis per hour

was plotted against temperature and is shown in Fig. 5.3. A temperature optimum of 33^{o}C was seen.

5.7. pH Dependance

The pH optimum of the poly (ADP-Ribose) glycohydrolase was determined using the same two buffers used in the polymerase pH dependance determination (4.6.). The standard assay was performed using exogenous [^{3}H] poly (ADP-Ribose). The results show that there appears to be two optima, one at pH 6.2, and the other at pH 8.0 (Fig. 5.4.).

5.8. Effect of β Mercaptoethanol

The effect of β mercaptoethanol on the glycohydrolase was examined by using a modified standard assay procedure. The modification used was to prepare the nuclei in a β mercaptoethanol-free medium then add appropriate amounts of β mercaptoethanol to the incubations. The results obtained are given in Table 5.2. and it can be seen that β mercaptoethanol has no effect on the glycohydrolase activity.

However, when DTNB was present in a twofold excess over the β mercaptoethanol in the standard assay, a 30% loss of glycohydrolase activity was seen.

5.9. Effect of Mg^{2+}

The effect of Mg^{2+} on the glycohydrolase was also examined in a similar manner to 5.8. (above). However, in this case the nuclei were prepared in Mg^{2+} free medium, appropriate aliquots of stock Mg^{2+} solution were added to the incubation mixtures. The results are given in Table 5.3. and show that

Fig. 5.3. Effect of varying temperature on the poly (ADP-Ribose) glycohydrolase activity in isolated wheat nuclei

Incubations carried out in 0.1M TEA-HCl, 2mM $MgCl_2$, 10mM β mercaptoethanol, pH 8.0. (100% c.p.m. per assay approx. 3000)

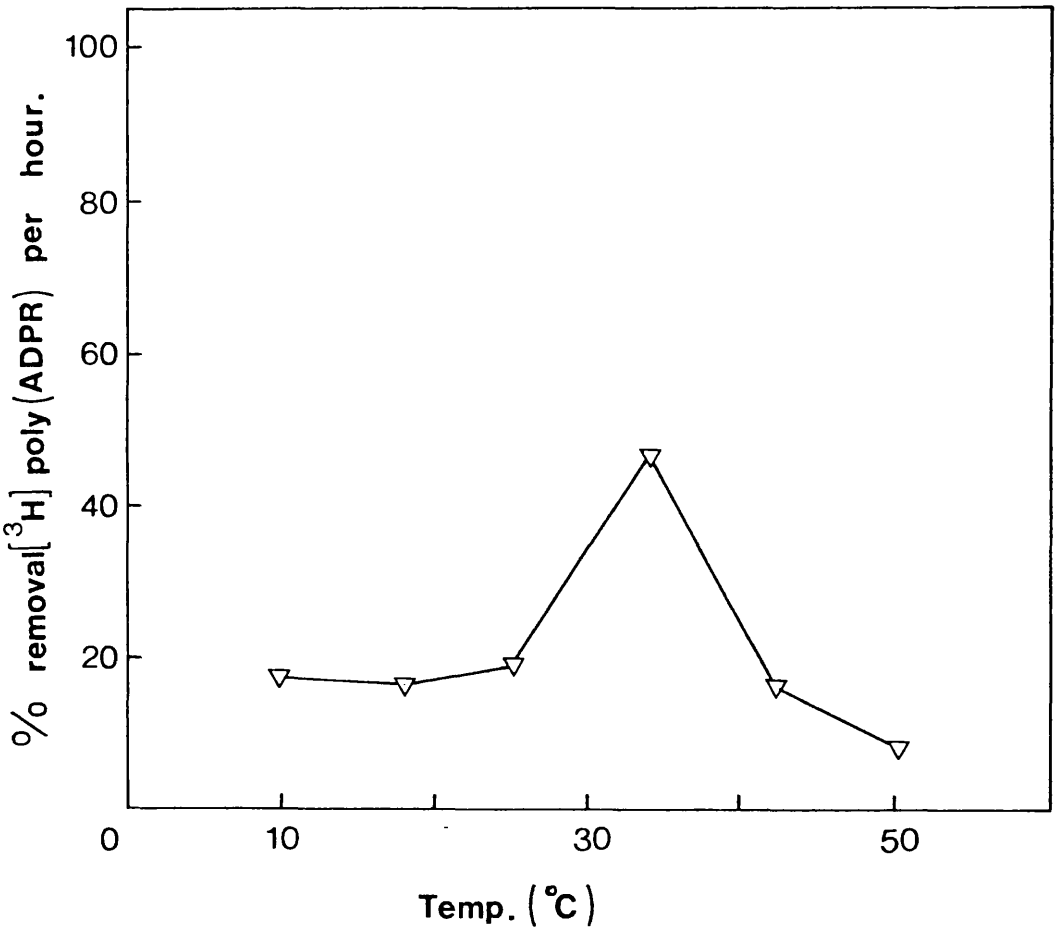

100
80
60
40
20
0
10
30
50
% removal[3H] poly(ADPR) per hour.
Temp. (℃)

Fig. 5.4. Effect of varying pH on poly (ADP-Ribose) glycohydrolase activity in isolated wheat nuclei

Incubations carried out at 26°C using either 0.1M Citrate-phosphate buffer (below pH 7.8) or 0.1M Tris-HCl buffer (pH 8.0 and above) as described in 5.7. (100% c.p.m. per assay approx. 3000)

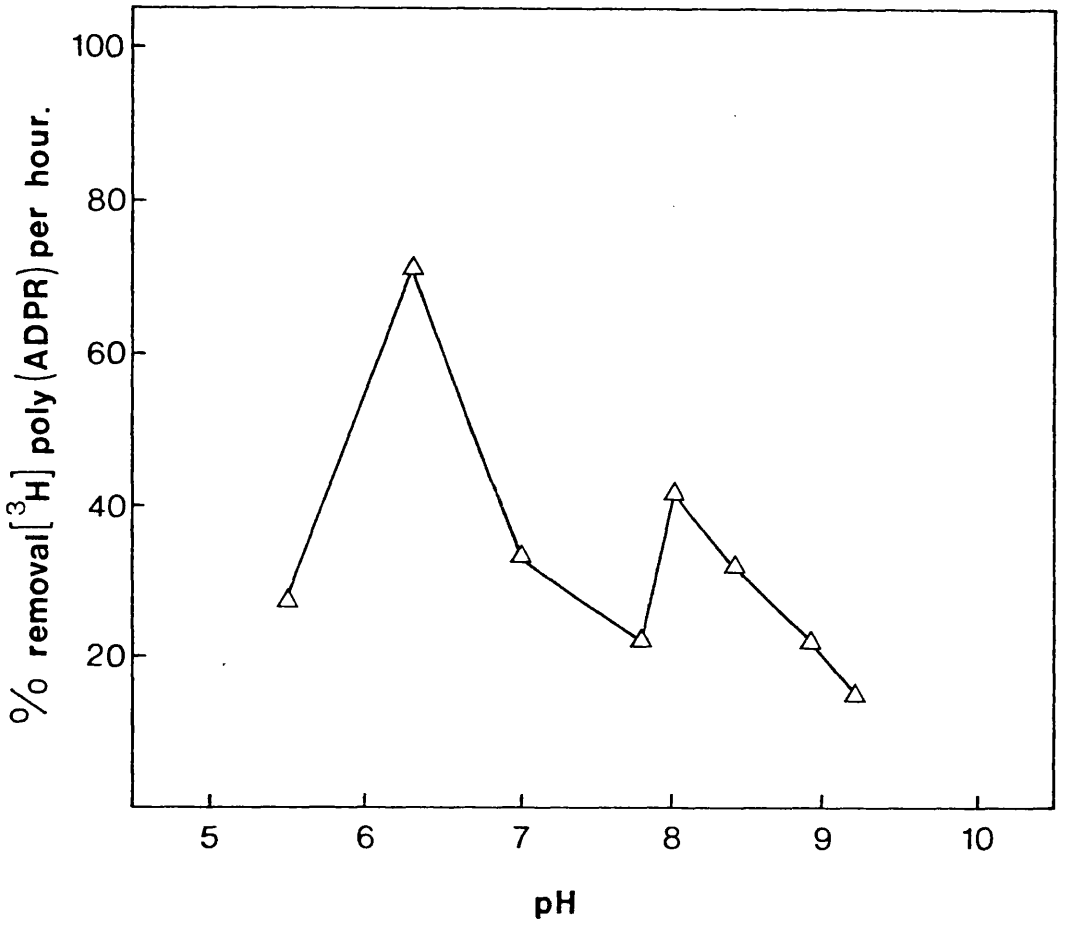

100
80
60
40
20
% removal [3H] poly (ADPR) per hour.
5
6
7
8
9
10
pH

TABLE 5.2. Effect of β mercaptoethanol upon the hydrolysis of [^{3}H] poly (ADP-Ribose) by a nuclear preparation isolated from wheat embryos

Concentration of mercaptoethanol (mM)	% acid insoluable cpm lost * per 10 minutes
0	8.38
1	8.52
2	8.56
5	9.13
10	8.80

* 100% cpm = approx. 10960

assay performed at 26°C in 0.1M TEA-HCl, 2mM $MgCl_2$ pH8.

TABLE 5.3. Effect of Mg^{2+} upon the hydrolysis of $[^3H]$ poly (ADP-Ribose) by a nuclear preparation isolated from wheat embryos

Concentration of $MgCl_2$ (mM)	% acid insoluble cpm lost * per 10 minutes
0	6.02
1	5.81
2	6.30
5	5.71
10	6.09

* 100% cpm = approx. 8698

assay performed at 26°C in 0.1M TEA-HCl, 10mM β mercaptoethanol pH8.

Mg^{2+} has no effect on the glycohydrolase activity.

5.10. Effect of Salts

Using the standard 'exogenous' assay, the effect of $(NH_4)_2\ SO_4$ and $NH_4\ Cl$ on the glycohydrolase activity was investigated. Appropriate amounts of crystalline salts were dissolved in the reaction mixture prior to the addition of $[^3H]$ poly(ADP Ribose). The results are shown in Fig. 5.5., showing that at concentrations above 0.5M of both, NaCl and $NH_4\ Cl$ are completely inhibitory.

5.11. Effect of Nucleotides and Polynucleotides

The same nucleotides and polynucleotides which were examined for their effect on the polymerase (4.10.) were also investigated for their effect upon the glycohydrolase. Of the compounds used, cyclic AMP, ATP, GTP, ADP-Ribose and single stranded DNA all proved inhibitory when used in the standard assay (Table 5.4.).

5.12. Concluding Remarks

The conclusions which can be drawn from the results presented above are as follows:-

(a) isolated wheat embryos contain poly (ADP-Ribose) glycohydrolase.

(b) the observed temperature optimum appears high when compared to most other reports which give a temperature optimum nearer the growth temperature of the cell source of the glycohydrolase (see 4.13b.). However, the glycohydrolase activity is detectable over a wide temperature range, which is in common with most

Fig. 5.5. Effect of Ammonium salts on poly (ADP-Ribose) glycohydrolase activity in isolated wheat nuclei

A = $(NH_4)_2\ SO_4$

B = NH_4Cl

Incubations carried out in 0.1M TEA-HCl, 2mM $MgCl_2$, 10 mM β mercaptoethanol (100% c.p.m. per assay approx. 2000)

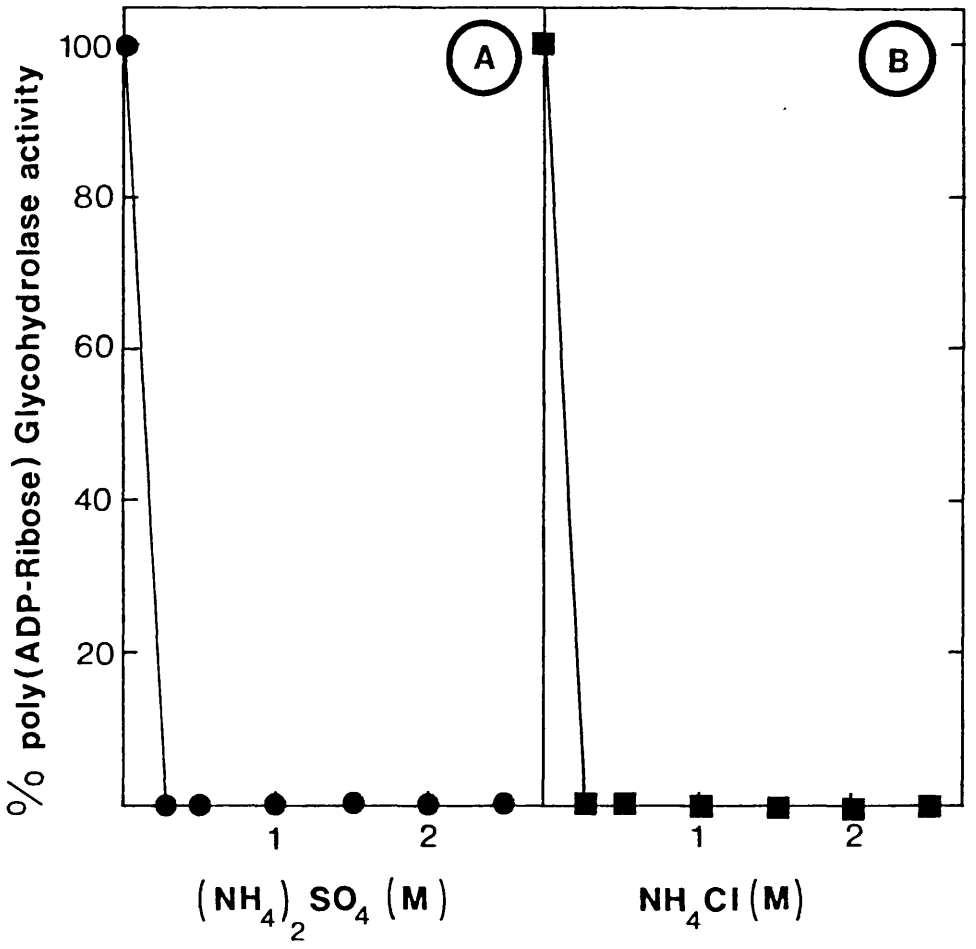

% poly(ADP-Ribose) Glycohydrolase activity
100
80
60
40
20
A
B
1
2
1
2
(NH4)2 SO4 (M)
NH4Cl (M)

TABLE 5.4. Effect of various compounds on poly (ADP-Ribose) Glycohydrolase activity in isolated wheat nuclei

Treatment	Poly ADP-Ribose degradation * (% polymer degraded per 10 min).
Control	17.0
1mM 5' AMP	12.5
10mM 5' AMP	1.8
1mM cyclic AMP	5.2
10mM cyclic AMP	0.3
1mM ATP	5.3
10mM ATP	0.3
1mM GMP	15.1
10mM GMP	14.0
1mM GTP	2.7
10mM GTP	0
1mM ADPR	2.1
10mM ADPR	0
10 µg.ml. ss DNA	0
10 µg.ml. ds DNA	10.5
10 µg.ml. RNA	8.9

* 100% $[^{3}H]$ poly (ADP-Ribose) = approx. 1,600 cpm per 10 µl aliquot

other studies (personal communication, P.R. Stone).

(c) Burzio et al (1975a, 1976) have demonstrated the existence of two variants of poly (ADP-Ribose) glycohydrolase in rat testis. These two variants, when examined in a partially purified enzyme preparation, gave two pH optima, pH 6.4 and pH 7.2. This study also gave two optima, pH 6.2. and pH 8.0. Although this result was repeatable the possibility that the effect was due to two different buffers cannot be ruled out.

(d) Both Mg^{2+} and β mercaptoethanol have no effect on wheat poly (ADP-Ribose) glycohydrolase. When nuclei were incubated with 5mM DTNB 11% of the glycohydrolase activity was lost. It was therefore concluded that the glycohydrolase does not have an absolute requirement for thiol groups.

(e) Both $(NH_4)_2\ SO_4$ and $NH_4\ Cl$ inhibit the wheat glycohydrolase. Miyakawa et al (1972) also reported that $(NH_4)_2\ SO_4$ inhibits the glycohydrolase from rat liver nuclei.

(f) The effect of the various nucleotides can be summarised as follows:- good inhibitors are cyclic AMP, ATP, GTP, ADP-Ribose and single stranded calf thymus DNA; poor inhibitors are AMP, GMP, native calf thymus DNA and RNA. Inhibition of poly (ADP-Ribose) glycohydrolase by cyclic AMP and ADP-Ribose has been reported by Miyakawa et al (1972) and Miwa et al (1974) amongst others (see Hilz and Stone, 1976, Hayaishi and Ueda, 1977). However, no reports have been published which show that ATP and GTP are inhibitors of the glycohydrolase. That ss DNA acts as an inhibitor suggests a possible in vivo mechanism of inhibition, which tends to lend support to ideas concerning the role of poly (ADP-Ribose) in DNA repair, when single stranded DNA is likely to be present (see Introduction, 1.2.).

An overall conclusion that can be drawn is that the wheat poly (ADP-Ribose) glycohydrolase is in many respects similar to glycohydrolases reported from other tissues (Hilz and Stone, 1976, Hayaishi and Ueda, 1977).

6. EFFECT OF POLYAMINES AND Mg^{2+} ON POLY (ADP-RIBOSE) SYNTHESIS AND ADP-RIBOSYLATION OF HISTONES

6.1. Introduction

In view of the effect of polyamines and Mg^{2+} in stimulating poly (ADP-Ribose) synthesis in isolated rat liver nuclei (Tanigawa et al, 1977, Perella and Lea, 1978, 1979), and the observation that plant tissues contain fluctuating levels of polyamines (Bagni, 1970, Ramakrisna and Adiga, 1974, Smith and Wilshire, 1975, Smith and Best, 1977, Villanueva et al, 1978), the effect of polyamines on poly (ADP-Ribose) synthesis in wheat nuclei was investigated.

6.2. Methods Employed

Nuclei were isolated from embryos as described above (2.5.). All incubations used the standard assay procedure outlined in 4.2., modified as noted below. The polyacrylamide gel electrophoresis was performed as described above (2.15.). The three polyamines examined were spermine, spermidine and putrescine.

6.3. Effect of Polyamines on ADP-Ribosylation

All three polyamines exhibited a dose-dependent effect in increasing the in vitro ADP-Ribosylation of isolated wheat nuclei (Fig. 6.1.a.). As described previously (4.8.) Mg^{2+} also shows a similar but somewhat smaller effect on ADP-Ribosylation (Fig. 4.6. and Fig. 6.1.b.). The stimulating effect of polyamines (at a concentration of 1mM) was shown in both the absence (Fig. 6.2.a.) and presence (Fig. 6.2.b.) of an optimal (2mM) Mg^{2+} concentration. These results are similar to the effects reported by Tanigawa et al, (1977).

Fig. 6.1. Effect of polyamine and Mg^{2+} concentration on ADP-Ribosylation in isolated wheat nuclei

A = polyamines, ○ = spermine,
● = spermidine; □ = putrescine.

B = Mg^{2+} , ▲ .

Incubations carried out at 26°C in 0.1M TEA HCl, 10mM β mercaptoethanol, pH 8.0.

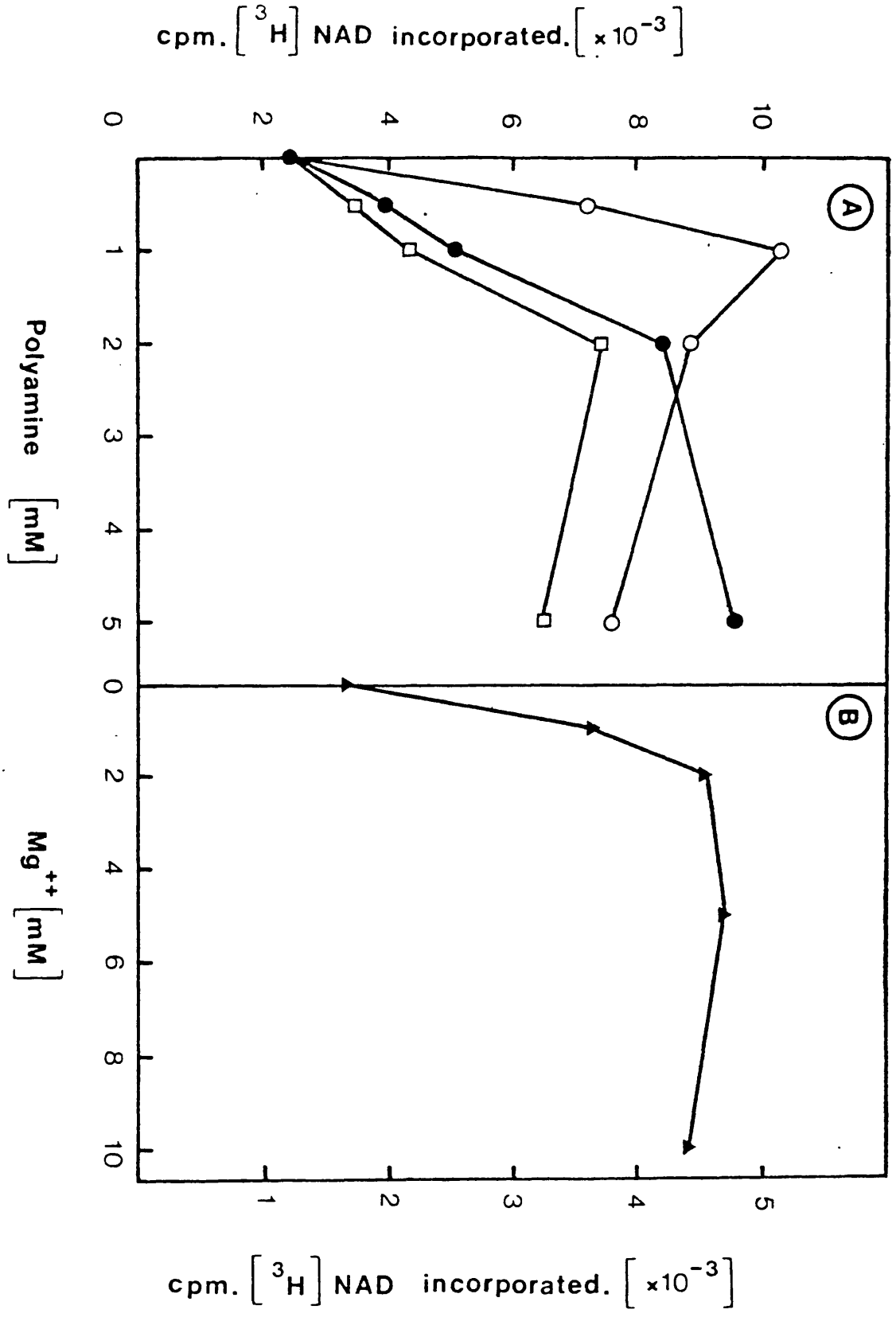

cpm. [³H] NAD incorporated. [×10⁻³]
0
2
4
6
8
10
A
Polyamine [mM]
1
2
3
4
5
0
B
Mg⁺⁺ [mM]
2
4
6
8
10
1
2
3
4
5
cpm. [³H] NAD incorporated. [×10⁻³]

Fig. 6.2. Effect of 1mM polyamines on the rate of ADP-Ribosylation in isolated wheat nuclei

A = in absence of Mg^{2+}

B = in presence of 2mM Mg^{2+}

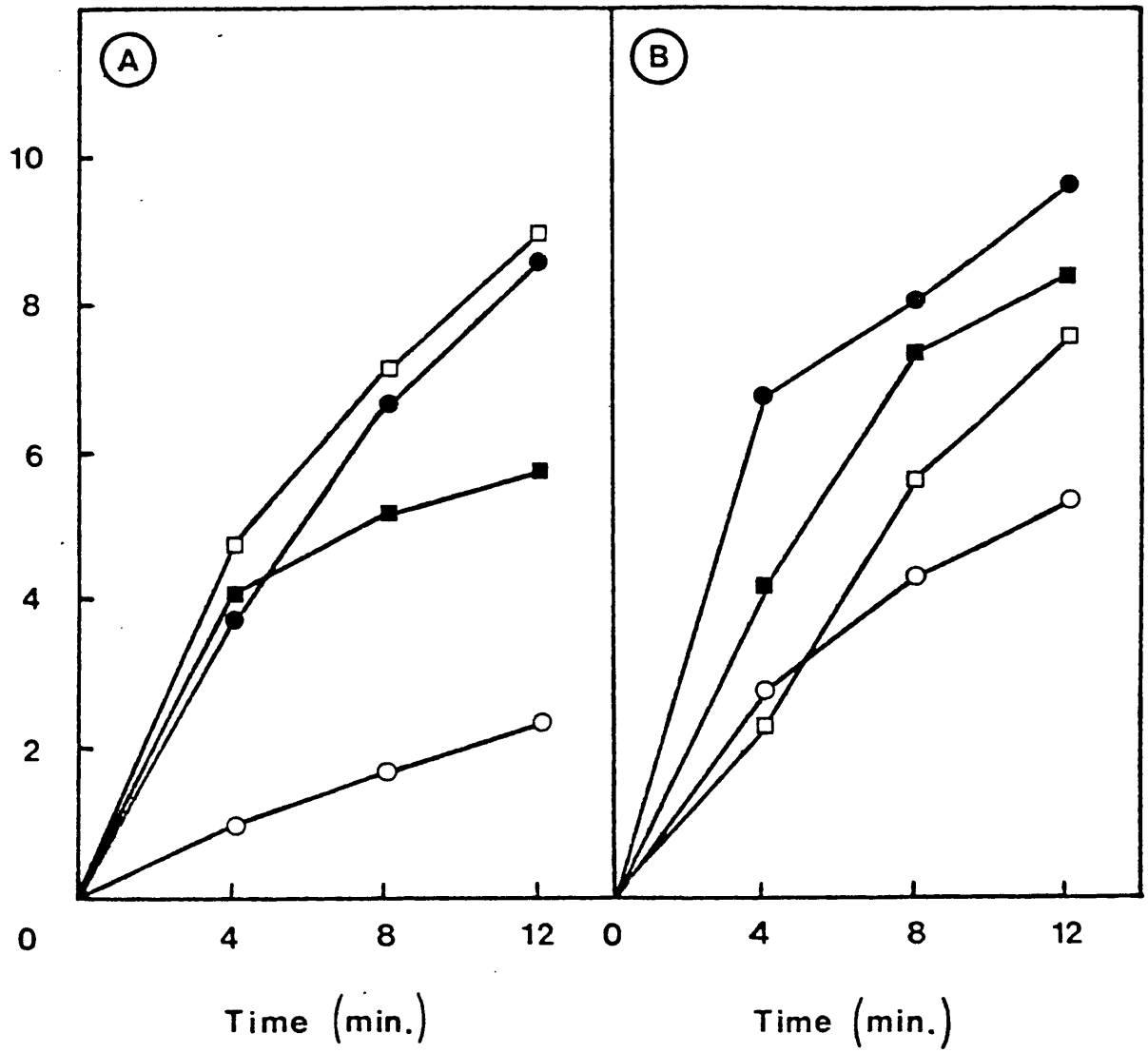
○ = control (no polyamine)

● = spermine

□ = spermidine

■ = putrescine

Incubations were carried out at 26°C in 0.1M TEA HCl, 10mM β mercaptoethanol, pH 8.0.


A
B
10
8
6
4
2
0
4
8
12
0
4
8
12
Time (min.)
Time (min.)

6.4. Fffect of Polyamines on Poly (ADP-Ribose) Glycohydrolase

There are several possibilities which may account for this observed increase in ADP-Ribosylation in the presence of Mg^{2+} and polyamines. One possibility is that the degradation of poly (ADP-Ribose) in the nuclei might be inhibited, resulting in an observed net increase in ADP-Ribosylation. To test this possibility the effect of polyamines on the poly (ADP-Ribose) glycohydrolase was investigated. The assay conditions employed are described in 5.2. Both the "endogenous" and "exogenous" glycohydrolase assays were used. The results are shown in Table 6.1. and it is clear that polyamines, in the presence and absence of Mg^{2+}, or Mg^{2+} alone, at concentrations which stimulate poly (ADP-Ribose) (Fig. 6.1. and Fig. 6.2.) do not inhibit the glycohydrolase. In fact there is some stimulation of the glycohydrolase by polyamines (Table 6.1.). This observation agrees with the report of Miwa et al (1974) who found that calf thymus poly (ADP-Ribose) glycohydrolase was stimulated by spermidine. The slower rate of polymer degradation obtained using exogenous poly (ADP-Ribose) is a result of the lower specific radioactivity of this material.

6.5. Effect of Polyamines on the Chain Length of Poly (ADP-Ribose)

A second possibility which may account for the observed increase in poly (ADP-Ribose) synthesised in the presence of polyamines and Mg^{2+} is that the average chain length of the polymer is increased. The average chain lengths of the poly (ADP-Ribose) synthesised in the presence and absence of polyamines and Mg^{2+} were examined as described previously (2.13.). The results are shown in Table 6.1. The values are all in very

TABLE 6.1. Effect of Polyamines and Mg^{2+} on the average chain length of poly (ADP-Ribose) in vitro

Treatment	Average Chain Length
Control	2.51
2mM $MgCl_2$	3.22
1mM Spermine	2.81
1mM Spermidine	3.10
1mM Putrescine	3.32
1mM Spermine + 2mM $MgCl_2$	2.88
1mM Spermidine + 2mM $MgCl_2$	3.14
1mM Putrescine + 2mM $MgCl_2$	3.41

all assay conditions are described in 6.2.

TABLE 6.2. Effect of Polyamines and Mg^{2+} on the degradation of poly (ADP-Ribose) by a nuclear preparation isolated from wheat embryos

Treatment	Poly (ADP-Ribose) degradation * (% polymer degraded per min)	
	Exogenous Assay	Endogenous Assay
Control	0.66	3.45
2mM $MgCl_2$	0.64	1.58
1mM Spermine	1.07	4.6
1mM Spermidine	1.02	6.46
1mM Putrescine	0.67	4.37
1mM Spermine + 2mM $MgCl_2$	0.87	4.78
1mM Spermidine + 2mM $MgCl_2$	0.84	6.08
1mM Putrescine + 2mM $MgCl_2$	0.91	3.87

* 100% $[^3H]$ poly (ADP-Riose) = 4000 cpm/10 µl aliquot in both cases

close agreement, indicating that Mg^{2+} and polyamines cause an increase in the number of polymer chains synthesised and have little, if any, effect on the average chain length of poly (ADP-Ribose) in isolated wheat nuclei.

6.6. Effect of Polyamines on the Distribution of ADP-Ribosylation of Histones

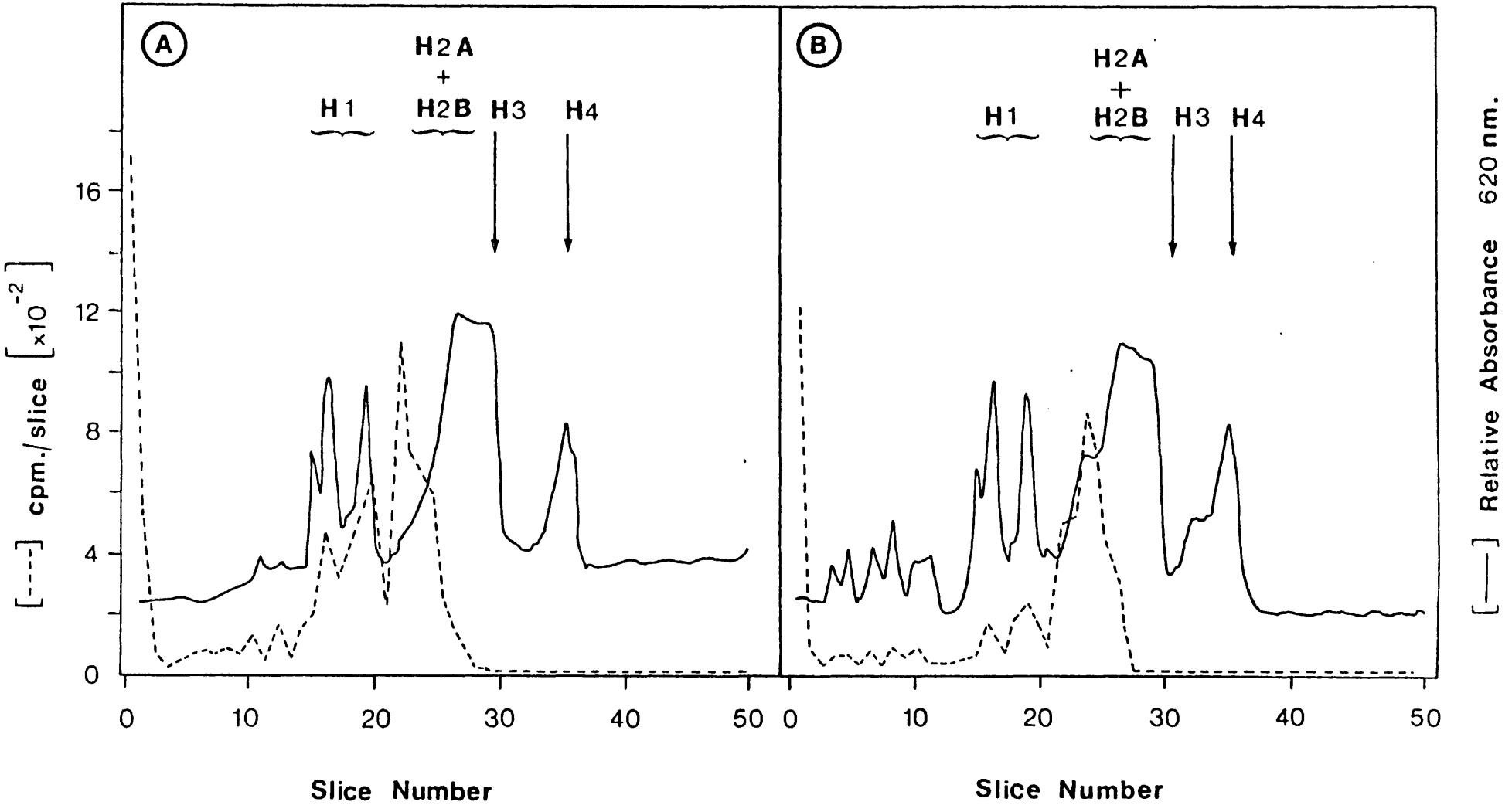
The possibility exists that the new poly (ADP-Ribose) chains synthesised in the presence of Mg^{2+} or polyamines may be preferentially associated with a specific nuclear protein fraction (e.g. histones, non-histone proteins). This seems to be the case in rat liver nuclei where it has been shown that ADP-Ribosylation occurs mainly in non-histones in the presence of 1mM spermine, while in the presence of Mg^{2+} ADP-Ribosylation occurs mainly in the histones (Tanigawa et al, 1977). Other workers have also observed an increased ADP-Ribosylation of non-histone proteins in the presence of both Mg^{2+} and spermine (Perella and Lea, 1978, 1979). To investigate whether this same effect occurs in wheat, isolated nuclei were incubated with $[^{3}H]$ NAD in the presence and absence of Mg^{2+} and polyamines. The histones were then extracted and analysed by polyacrylamide gel electrophoresis. Representative gel profiles are shown in Fig. 6.3.a. for histones isolated from chromatin, and Fig. 6.3.b. for histones isolated from nuclei. The two profiles shown are from incubations in the absence of both Mg^{2+} and polyamine. These results indicate that histones H1, H2A and H2B are heavily modified. In the case of H2A and H2B the ADP-Ribose profile does not exactly coincide with the protein profile. This is not too surprising since heavily ADP-Ribosylated

Fig. 6.3. Distribution of [^{3}H] poly (ADP-Ribose) on acetic-acid-urea polyacrylamide gels

A = sample from chromatin preparation

B = " " isolated nuclei

Each sample was incubated with [^{3}H] NAD at 26°C in 0.1M TEA HCl, 2mM Mg Cl_2, 10mM β mercaptoethanol pH 8.0.


A
B
H2A
+
H2B
H1
H3
H4
[---] cpm./slice [x10^-2]
[——] Relative Absorbance 620 nm.
0
4
8
12
16
0
10
20
30
40
50
Slice Number

histone H1 electrophoreses slower than unmodified H1. (Lorimer et al, 1977, Stone et al, 1977, Tanuma et al, 1977, Byrne et al, 1978, Perella and Lea, 1978, 1979). There is no modification of histone H4 and little or no modification of histone H3.

Similar gel analyses were performed on histones extracted from nuclei incubated with [^{3}H] NAD in the presence and absence of Mg^{2+} and polyamines. The results are summarised in Table 6.3. and it can be seen that the stimulation of total poly (ADP-Ribose) synthesis is accompanied by a similar increase in the total histone associated poly (ADP-Ribose). These results differ from those obtained in rat liver, (Tanigawa et al, 1977) showing that in wheat nuclei there is no preferential ADP-Ribosylation of histones caused by Mg^{2+} and polyamines. It can also be seen that the inter-histone ratios remain fairly constant when incubated in the presence of Mg^{2+} and polyamines. This is again different to the situation in rat liver nuclei where it has been shown that 1mM spermine causes no change in the extent of ADP-Ribosylation of total histones although a redistribution of the ADP-Ribose from the core histones to H1 was observed (Perella and Lea, 1978, 1979).

6.7. Concluding Remarks

Whether such discrepancies seen between the wheat and rat liver systems reflect inherent differences in the ADP-Ribosylation machinery is unclear. It may be that the observed differences are solely due to the unique features of the wheat acceptor proteins (H1, H2A, H2B). However, in view of the observed ADP-Ribosylation of these histones in isolated wheat

TABLE 6.3. Distribution of $[^3H]$ poly (ADP-Ribose) in the Histone fraction isolated from a nuclear preparation from wheat embryos

distribution determined by acid urea - polyacrylamide gel electrophoresis

Treatments	Total Acid Insoluble cpm	Total Histone Fraction		Histone H1 as % total histone	Histones H2A/H2B as % total histone
		cpm	% of total		
Control	76,562	10,420	13.6	21	79
2mM $MgCl_2$	200,581	37,660	18.8	26	74
1mM Spermine	209,156	45,360	21.7	20	80
1mM Spermidine	192,762	32,890	17.1	19	81
1mM Putrescine	136,990	26,330	19.2	24	76

nuclei and the stimulation by polyamines, it may well be that ADP-Ribosylation of histones in plant cells is involved in extending or condensing chromatin, as has been postulated for mammalian cells (Stone et al, 1977, Lorimer et al, 1977, Byrne et al, 1978, Perella and Lea, 1978, 1979).

7. POLY (ADP-RIBOSE) DURING GERMINATION

7.1. Introduction

The germination of seeds provides a good model system for the study of metabolic events, in particular nucleic acid metabolism (for review see Bryant, 1976). This work is a preliminary study of poly (ADP-Ribose) during the early stages of germination. These investigations have shown that cycloheximide sensitive protein synthesis commences approximately 1-2 hours after the onset of imbibition; DNA and RNA synthesis commence approximately 14 hours after the onset of imbibition.

7.2. Methods

Wheat embryos were allowed to imbibe and germinate using the following procedure:- Approximately 1.5 g dry wheat embryos were placed on a filter paper disc. The paper disc and embryos were moistened with 1% (w/v) glucose, 0.01% (w/v) streptomycin sulphate, covered and incubated in the dark at 26^{o}C. At various time intervals, nuclei were prepared from the embryos, as described in 2.5. The nuclei were then assayed for poly (ADP-Ribose) polymerase and glycohydrolase activities. The results were expressed as cpm incorporated or removed per mg. DNA, the DNA being measured by the method of Setaro and Morley (2.8.).

7.3. Poly (ADP-Ribose) Polymerase and Glycohydrolase during Germination

The polymerase activity was determined using the standard assay described in 4.2. The glycohydrolase activity was determined using the standard assay employing 'exogenous' substrate

as described in 5.2. The variations of these activities during imbibition are shown in Fig. 7.1. and Fig. 7.2. respectively. Each point is the mean $\pm$ standard deviation of a minimum of 5 separate experiments. The time given is from the onset of imbibition since it is impossible to separate imbibition from germination.

7.4. Effect of Na Cl on Glycohydrolase activity

The apparent loss of poly (ADP-Ribose) glycohydrolase activity after 6 hours of imbibition (Fig. 7.2.) was further investigated. Two possible explanations were considered:- (a) a reorganisation of the chromatin was occurring, resulting in a reduction of enzyme activity and (b) the glycohydrolase was being inhibited by an inhibitor synthesised *in vivo*. A third possibility, not examined in this study, is that the poly (ADP-Ribose) glycohydrolase was being subjected to proteolysis.

The first possibility was examined by incubating nuclei in the presence of high (up to 0.5M) concentrations of Na Cl. Nuclei were prepared from dry embryos as described in 2.5. Aliquots of these nuclei were mixed with varying amounts of Na Cl and then allowed to stand on ice for 15 minutes. Prior to measuring the glycohydrolase activity all samples were made to a final concentration of 0.1M with respect to Na Cl. The glycohydrolase activity was measured using the standard assay, employing exogenously supplied [^{3}H] poly (ADP-Ribose) (5.2.). As can be seen (Table 7.1.), preincubating with 0.5M Na Cl released approximately 60% more glycohydrolase activity when compared to the control (no Na Cl). Having shown that Na Cl

Fig. 7.1. Changes in poly (ADP-Ribose) polymerase activity during germination

embryos germinated in 1% (w/v) glucose, 0.01% (w/v) streptomycin sulphate at 26°C in the dark
(Results corrected for glycohydrolase activity.)

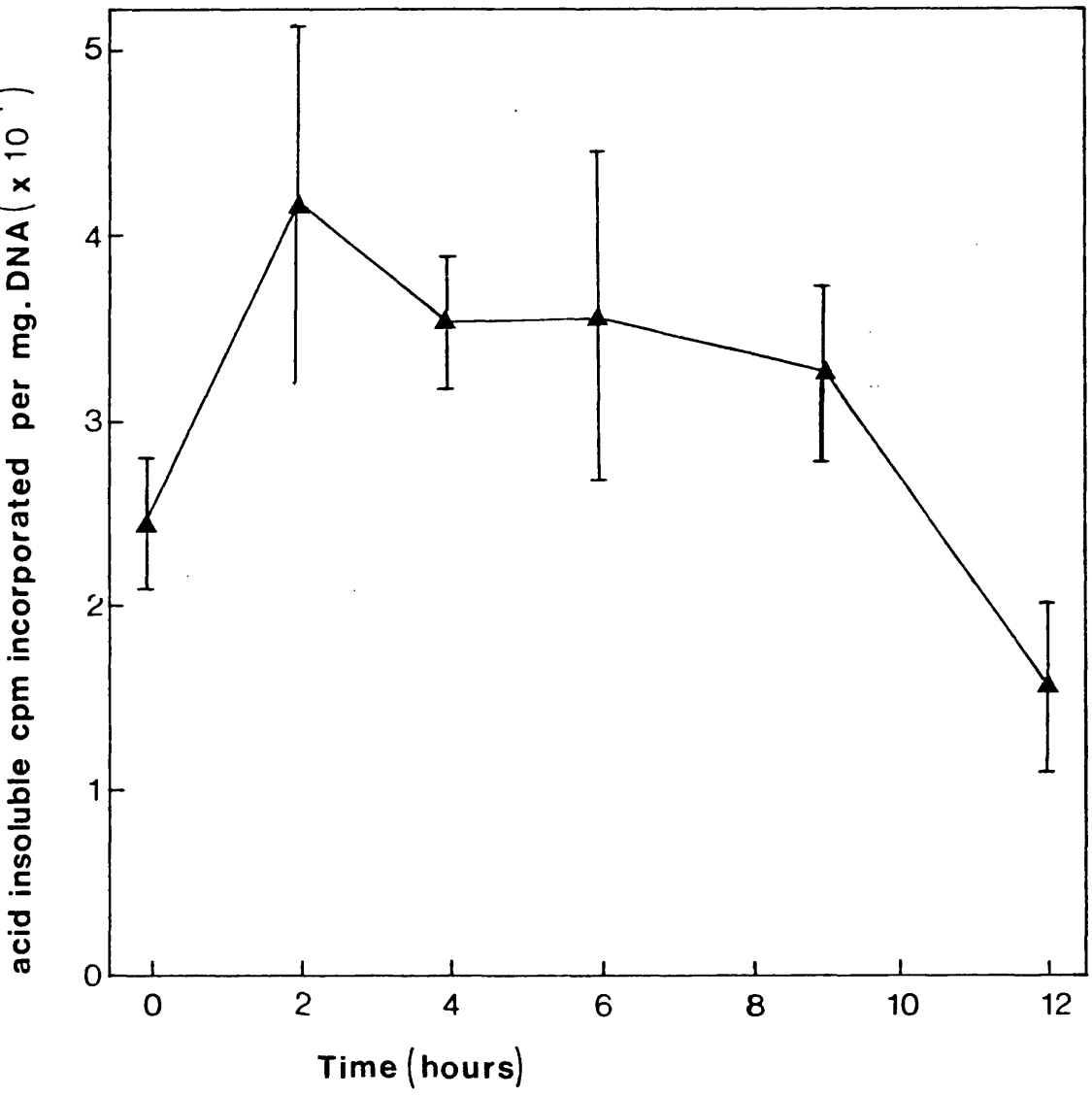

acid insoluble cpm incorporated per mg. DNA (x 10^{-4})
0
1
2
3
4
5
0
2
4
6
8
10
12
Time (hours)

Fig. 7.2. Changes in poly (ADP-Ribose) glycohydrolase activity during germination

embryos germinated in 1% (w/v) glucose 0.01% (w/v) streptomycin sulphate at 26^{o}C in the dark

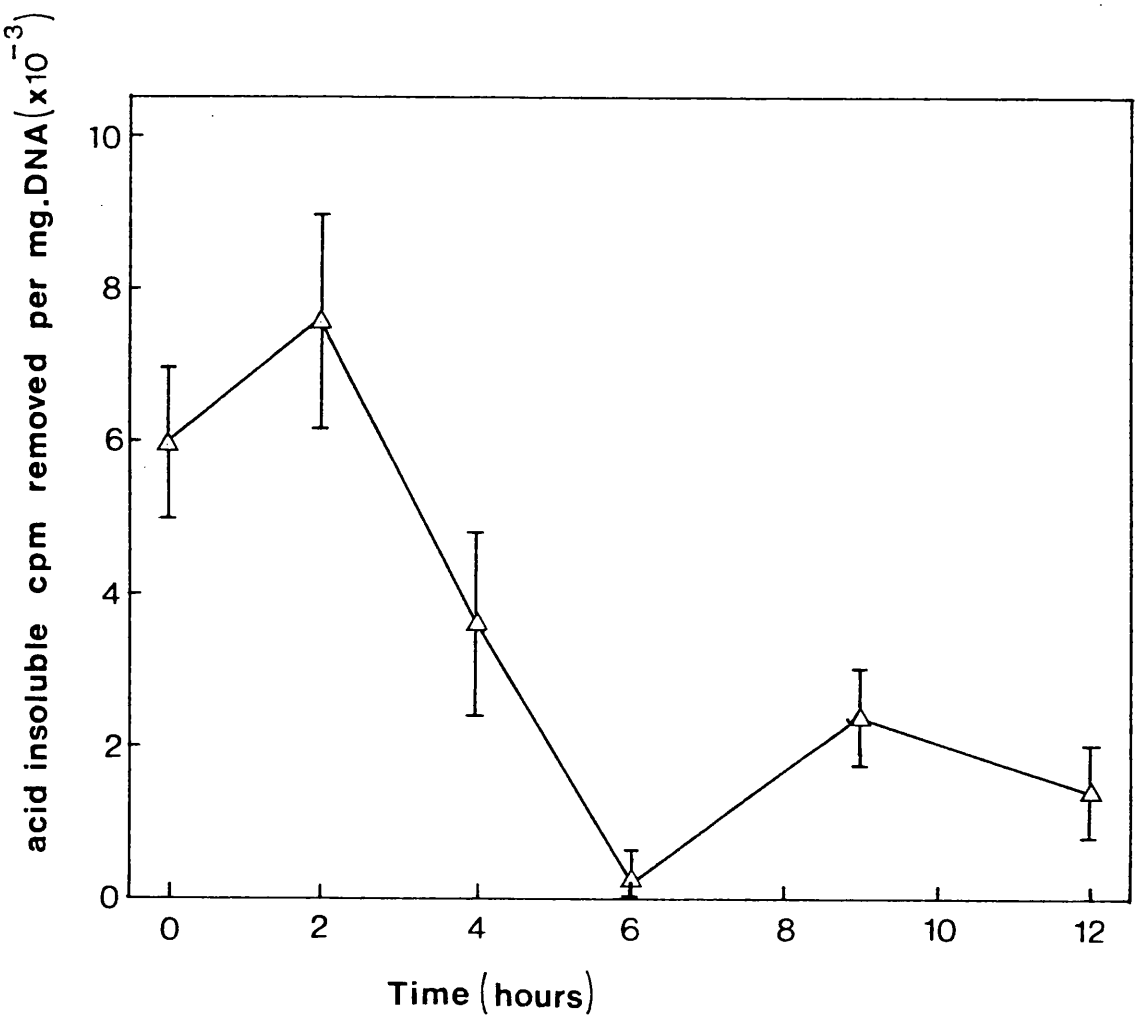

acid insoluble cpm removed per mg.DNA (x10^{-3})
10
8
6
4
2
0
0
2
4
6
8
10
12
Time (hours)

Table 7.1. Effect of increasing NaCl concentrations on poly (ADP-Ribose) glycohydrolase in isolated wheat nuclei

Concentration NaCl (M)	Relative activity *
0	3.56
0.05	3.86
0.1	4.27
0.25	5.55
0.5	5.59

* activity expressed as % acid insoluble cpm degraded per minute; 100% = approx. 6000 cpm.

is not inhibitory to the glycohydrolase at concentrations up to 0.5M nuclei were prepared from embryos which had imbibed in the dark for 6 hours at 26°C. No difference was observed in the glycohydrolase activity between a control (adding an equivalent volume of buffer) and a sample treated with 0.5M Na Cl. It was therefore concluded that the loss of glycohydrolase activity was not due to condensation effects in the chromatin.

7.5. An inhibitor of poly (ADP-Ribose) Glycohydrolase *In Vivo*

The presence of an inhibitor was investigated as follows. It was decided that the best way to demonstrate the presence of an inhibitor would be to mix nuclei prepared from dry embryos with a preparation from embryos which had imbibed for 6 hours. A further control was employed which used a preparation from embryos which had undergone 2 hours imbibition (i.e. where a significant amount of glycohydrolase activity was observable). A salt extract of the nuclei was prepared by making the nuclei 0.5M with respect to Na Cl. The mixtures were then allowed to stand on ice for 30 minutes, followed by centrifugation at 35,000 rpm at 4°C for 30 minutes, using a Beckman L5 ultra-centrifuge and a 50 Ti angle rotor. The pellet was discarded and the supernatant termed the salt extract. Prior to use the salt extract was diluted to 0.1M Na Cl. Salt extracts were prepared from embryos which had been imbibing for 2 hours and 6 hours and a nuclear preparation was also made from dry embryos. The following incubations were carried out together with a control which contained salt solution but no salt extract:-

(a) nuclei + equivalent volume of salt solution.

(b) nuclei + salt extract from '2 hour' embryos.

(c) nuclei + salt extract from '6 hour' embryos.

The glycohydrolase activity was measured by using the standard assay and exogenous [^{3}H] poly (ADP-Ribose) (5.2.). The results are shown in Fig. 7.3.a. for experiments (a) and (b); Fig. 7.3.6. shows (a) and (c). As can be seen the salt extract from embryos imbibed for 2 hours has no effect on the glycohydrolase activity. However, a salt extract prepared from embryos imbibed for 6 hours is completely inhibitory.

The above results indicate that germinating wheat embryos synthesise a potent inhibitor of poly (ADP-Ribose) glycohydrolase in vivo. The nature of this inhibitor has yet to be elucidated, however, the following points are worth noting. It is possible that the inhibitor is either a protein or nucleic acid fragment as both these classes of material are present in the supernatant following the centrifugation step in the preparation of the salt extract. As noted above (Table 5.4.) various nucleotides (e.g. ATP, GTP) inhibit the glycohydrolase at high concentrations (10mM). Several studies (see 1.3.) have shown that phosphorylated nucleotide levels do fluctuate during seed germination, however, it is unlikely that the isolated nuclei will contain sufficient nucleotide to give the observed loss of activity after the embryos have been allowed to imbibe for 6 hours. Another potential candidate (from Table 5.4.) is single stranded DNA. This is however a remote possibility; the preparation of the nuclei involves sonication which will give rise to some ss DNA. If ss DNA is the inhibitor it would then be necessary to postulate a mechanism whereby the DNA is much more sensitive to breakage by sonication after

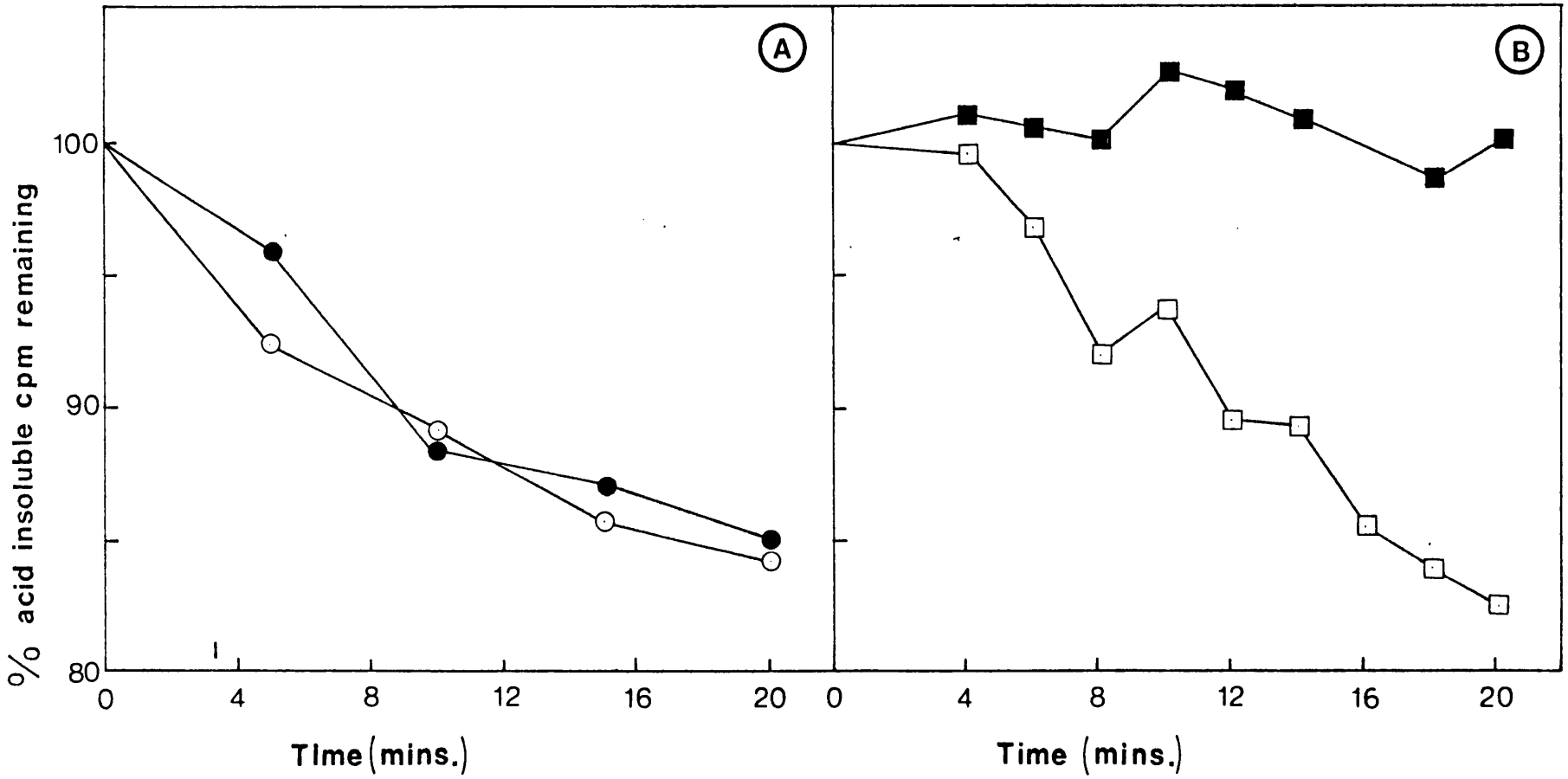
Fig. 7.3. Effect of mixing experiments upon poly (ADP-Ribose) glycohydrolase activity

(a) ○ = nuclei prepared from dry embryos + 0.1M NaCl

● = nuclei prepared from dry embryos + salt extract from embryos that had imbibed for 2 hours

(b) □ = nuclei prepared from dry embryos + 0.1M NaCl

■ = nuclei prepared from dry embryos + salt extract from embryos that had imbibed for 6 hours


A
B
100
90
80
0
4
8
12
16
20
% acid insoluble cpm remaining
Time (mins.)
Time (mins.)

the embryos have imbibed for 6 hours.

Further studies investigating the nature of the inhibitor are under consideration in this laboratory. Some of the ideas being examined include:-

(a) allowing the embryos to imbibe in the presence of cycloheximide (thereby inhibiting protein synthesis). This approach should determine whether or not the inhibitor is a polypeptide or protein synthesised de novo.

(b) dialysis of a salt extract made from '6 hour' embryos. By using different exclusion limit dialysis membranes an idea of the molecular size of the inhibitor may be obtained.

(c) a better proposition than (b) would be to use gel filtration. If an active fraction can be obtained classical analytical techniques could then be employed.

(d) attempting to inhibit the various stages of transcription and translation, to determine if the inhibitor is either a nucleic acid (e.g. m RNA) or the product of gene expression, either from stored m RNA or newly synthesised m RNA.

8. GENERAL DISCUSSION

The polymerase, glycohydrolase and histone modifications are discussed in detail in the relevant chapters (above). As an overall view the poly (ADP-Ribose) system in germinating wheat seeds appears to be similar to the animal systems studied. It would therefore seem reasonable to conclude that the function(s) of poly (ADP-Ribose) might be common to both plants and animals.

Owing to the rather incomplete knowledge of plant biochemistry compared to animal systems, it is difficult to draw direct analogies between the two. However the present study has provided pointers to several roles for poly (ADP-Ribose). The first of these concerns the involvement of poly (ADP-Ribose) in DNA repair. This topic is currently receiving considerable attention in several laboratories (see 1.2.). It has been shown that radiation of sufficient intensity to cause damage to cellular DNA (e.g. 800 rads) also causes a significant lowering of cellular NAD levels (Scaife, 1963). Similar results have been obtained by treating cells with alkylating reagents, which cause chemical modification of DNA. (Schein et al, 1967). In both cases the drop in cellular NAD levels has been correlated with an increased flux through poly (ADP-Ribose) (Whish et al 1975, Davies et al 1978a, Davies et al 1978b, Skidmore et al 1979). Two main types of DNA repair are known:- excision repair and post-replication repair. Excision repair consists of removal of a damaged segment of single stranded DNA (e.g. a thymine-thymine cyclobutane dimer, caused by U.V. irradiation) via a damage specific endonuclease. The resultant 'gap' is filled via a DNA polymerase and then sealed by a DNA ligase. Post-replication repair involves a

similar mechanism, but occurs after cellular DNA replication. It is believed that cross-linking of DNA strands by bifunctional alkylating agents is removed by a similar mechanism. A full review of DNA repair can be found in Lehmann and Bridges, 1977. The present study has shown that single stranded DNA is a potent inhibitor of wheat poly (ADP-Ribose) glycohydrolase (Table 5.4.). Single stranded DNA is present during the excision repair, following the excision, and prior to DNA polymerase activity. Inhibition of the glycohydrolase would lead to an apparent stimulation of poly (ADP-Ribose) polymerase activity, assuming that both enzymes are normally acting at the same time, and the degree of ADP-Ribosylation is determined by the net rate of synthesis. However, Smulson and colleagues have shown that the increased formation of ADP-Ribose found in He La cells treated with alkylating agents is not due to a decrease in glycohydrolase activity (Sudhakur et al, 1979a, 1979b). The observation by Yoshihara et al (1975) that Ca^{2+} Mg^{2+} dependent endonucleases from liver and semen are reversibly inhibited by ADP-Ribosylation, may also hold for wheat. If so, the inhibition of the glycohydrolase by ss DNA would result in the inhibition of such an endonuclease, thereby terminating the initial stage of DNA repair i.e. the excision.

The second possibility involves the potential link between cellular NAD levels and the degree of poly (ADP-Ribose) formation in vivo. Ghani and Hollenberg (1978a, 1978b) have suggested that the regulation of cardiac cell division by oxygen is facilitated, at least in part, by altering the redox state of NAD ($NAD^+ \rightleftharpoons NADH$). Lower partial pressures of oxygen, which increase the rate of cell divison, decrease poly

(ADP-Ribose) formation by increasing the NADH levels, thereby reducing the amount of available NAD. Conversely, higher partial pressures of oxygen, which decrease the rate of cell division, would cause more NAD to be available, and hence more poly (ADP-Ribose). They suggest that poly (ADP-Ribose) participates in cell division by one or more mechanisms. This study has shown (chap. 7.) that there is a marked decrease in poly (ADP-Ribose) glycohydrolase activity during germination, whilst the polymerase activity did not significantly change. Preliminary work in this laboratory (Brown 1979) has shown that fluctuations in the NAD levels of germinating wheat seeds are inversely related to changes in poly (ADP-Ribose) glycohydrolase activity. These results imply that during wheat embryo differentiation the NAD levels may be controlled in part by the formation and degradation of poly (ADP-Ribose). However, this possibility appears to be energetically wasteful if the sole function of poly (ADP-Ribose) is to regulate NAD levels. For further details of NAD metabolism see 1.3. and Fig. 1.3.

The wheat system is a good model, several aspects of which could be further utilised to investigate the role of poly (ADP-Ribose). The techniques employed in this study can be used to continue manipulation of the system in vitro. In particular, if the glycohydrolase inhibitor postulated in chapter 7 can be isolated, it will no longer be necessary to examine the net synthesis of poly (ADP-Ribose) because until now no effective glycohydrolase inhibitor has been described (see Hilz and Stone 1976, Purnell et al 1980). The possibility also exists that a more detailed examination of histone modification might give a useful insight into the function of

ADP-Ribosylation. Such a study might include a determination of which histones in the nucleosome are modified and how this affects chromatin condensation, together with a comparison with data obtained from animal cells. Another approach will be to examine ADP-Ribosylation in vivo. Recent reports by workers in this laboratory (Surowy and Whish 1980, Whish and Poirer 1980) have demonstrated formation of ADP-Ribosylated proteins in vivo. Preliminary work has indicated that similar techniques can be modified for use with isolated wheat embryos (personal communication N.Abed). It is in this area that the postulated glycohydrolase inhibitor may be of more use, as some of the other compounds shown to be inhibitory (e.g. cyclic AMP, ATP) exert other metabolic effects. A further possibility for utilising the wheat system exists, that is to purify the polymerase. This could be done by exploiting the reversible inhibition of the wheat polymerase by Hg^{2+}. By coupling Hg^{2+} to an inert support (e.g. activated sepharose) the polymerase, and other -SH containing proteins, could be isolated by simply running a nuclear preparation over the column. The bound proteins could then be eluted from the column using β mercaptoethanol. This would give a simple, very rapid, method of preparing poly (ADP-Ribose) polymerase which was free of DNA, RNA and some, if not all, other proteins. This method of purification would help resolve the question of whether the polymerase needs DNA for maximal activity (see Hilz and Stone, 1976).

Note added in proof

Since the completion of this study several reports relating to poly (ADP-Ribose) have been published. Three of these are of particular significance to this report. The first report, by Durkacz *et al* (1979) deals with a function of poly (ADP-Ribose) *in vivo*. These workers have shown that $(ADP\text{-}Ribose)_n$ biosynthesis is required for efficient DNA excision repair and consequent survival following treatment of mouse L1210 cells with mono functional alkylating agents. The second report concerns the characterisation of poly (ADP-Ribose) polymerase activity in BHK cells (Furneaux and Pearson 1980). They have shown that the polymerase has very similar properties to those reported for other systems. Of particular interest, in view of this study, is that a biphasic deviation from normal Michaelis-Menten kinetics of the polymerase was observed, leading the authors to postulate the presence of two (or more) enzymes possessing poly (ADP-Ribose) polymerase activity. This finding can be seen as tentative confirmation of similar results obtained in this study (see discussion of Chapter 4). The third report of significance is that of Willmitzer (1979), demonstrating that the chromosomal proteins in transformed tissue cultures of Nicotinia tobaccum were covalently modified by poly (ADP-Ribose). These cultures exhibited a marked similarity to previously described animal systems, with an average polymer chain length of 3.4 to 8.6. The principle chromosomal acceptors were histones H1, H2A and H2B. These results are in very close agreement with results given above (Chapter 6) and published elsewhere (Whitby *et al* 1979, see appendix.)

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APPENDIX

Publications arising from this study are:-

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Poly(Adenosine Diphosphate Ribose) in Wheat

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The wheat seed used was stored at 4±1°C until required and before use was washed with approx. 2 litres of distilled water per 100g of seed. The seeds were germinated in the dark on moist filter papers at 20°C±1°C.

The extraction of chromatin from the ungerminated seeds was based on that of Simon & Becker (1976). Approx. 25g of dry seed was washed, then homogenized in approx. 100ml of extraction buffer 1 [0.05M-Tris/HCl (pH8.1)/15mM-β-mercaptoethanol/0.5% (v/v) Triton X-100] for 10min in a Sorval homogenizer. The homogenate was filtered through two layers of muslin, the retained material was washed with 20ml of extraction buffer 1 and then stirred for 10min at 4°±1°C. All subsequent steps were carried out at this temperature. The filtered homogenate was centrifuged at 4000*g* for 5min, the precipitate was discarded and a small volume of 3M-$(NH_4)_2SO_4$ was added, sufficient to make the supernatant 0.05M with respect to $(NH_4)_2SO_4$. The supernatant was recentrifuged at 10000*g* for 10min. The resulting supernatant was discarded. The pellet was resuspended in 50ml of extraction buffer 2 [0.05M-Tris/HCl (pH8.1)/15mM-β-mercaptoethanol/0.05M-$(NH_4)_2SO_4$/0.5% (v/v) Triton X-100] by using a Teflon pestle; the resulting homogenate was centrifuged at 10000*g* for 10min, and the pellet made up in 50ml of extraction buffer 3 [0.01M-Tris/HCl (pH8.1)/15mM-β-mercaptoethanol/2mM-$MgCl_2$]. This was recentrifuged at 10000*g* for 10min and the final pellet made up in a small volume of incubation buffer [0.1M-Tris/HCl (pH8.2)/0.06M-KCl/0.01M-$MgCl_2$/0.004M-KF)].

Nuclei were prepared by harvesting the root tips of 4–7-day-old germinated seeds, taking approx. 2g of damp material and homogenizing in 5ml of extraction buffer 4 [0.05M-Tris/HCl (pH7.0)/0.25M-Sucrose/0.01M-$MgCl_2$/0.1% (v/v) Triton X-100] on ice in a Braun homogenizer. The homogenate was filtered through one layer of muslin, washed with a small volume of extraction buffer 4 and the filtrate was centrifuged at 2000*g* for 10min at 4°±1°C. The supernatant was discarded, and the nuclei in the pellet were counted by using a Neubauer haemocytometer.

The protein content of the samples was measured by the procedure of Bramhall *et al.* (1969) and the DNA content was measured by the modified procedure of Burton (1968).

The poly(ADP-ribose) activity was investigated by the following assay. A 200μl sample in incubation buffer was taken; to this was added 20μl of 0.1M-dithiothreitol and the reaction started by the addition of 5μl of [^{3}H]NAD (20mCi/μmol; 0.05mM). It was incubated for 10min at 25°C and the reaction stopped by the addition of 2ml of 20% (w/v) trichloroacetic acid and the solution placed on ice for 30min. The solution was then filtered with a Millipore GF/C filter and washed with 1% (w/v) trichloroacetic acid; the paper was then placed in a small (50mm×12mm) glass tube and dried at 100°C. When dry, sufficient scintillant (0.5%, w/v, 2,5-diphenyloxazole in toluene) to cover the paper was added, and the whole was placed in a scintillation vial and counted for radioactivity.

The results showed that the amount of acid-insoluble [^{3}H]NAD incorporation in the non-germinated seeds was approximately double that of the nuclei isolated from roots of germinated seeds, taken with respect to both protein and DNA. The incorporation was markedly inhibited in both cases by nicotinamide. The acid-insoluble incorporation was shown to be poly(ADP-ribose) by using t.l.c. and enzymic digestion (Whish *et al.*, 1973). The poly(ADP-ribose) was hydrolysed by snake-venom phosphodiesterase which gave rise to the unique compound phosphoribosyl-AMP. The latter was characterized by further digestion with alkaline phosphatase to give ribosyladenosine. These compounds were identified by standard t.l.c. techniques. The chain length of the polymer was estimated at the same time by measuring the ratio of AMP counts to total polymer counts.

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Poly(Adenosine Diphosphate Ribose) Polymerase and Deoxyribonucleic Acid Damage

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Several classes of cytotoxic agents are known to affect glycolysis in cells by lowering the cellular concentration of NAD. Early investigations showed that both alkylating agents (Roitt, 1956) and ionizing radiation (Maass *et al.*, 1958) were capable of producing this effect, and subsequent research has largely been confined to these agents.

Most of the NAD turnover is confined to the cell nucleus; only 5% of the NAD synthesized is required to maintain the cellular pool during growth (Rechsteiner *et al.*, 1976*a*,*b*). The rest is needed to replace that degraded, presumably by the DNA-dependent chromosomal enzyme poly(ADP-ribose) polymerase.

We have investigated the relationship of this enzyme to the decrease in NAD after treatment with cytotoxic drugs. The activity of the enzyme increases twofold on treatment of *Physarum polycephalum* with the alkylating agent streptozotocin (Whish *et al.*, 1975). Inhibition of the enzyme has been shown to prevent the decrease in NAD caused by streptozotocin in mouse leukaemia cells (Davies *et al.*, 1976). We have now extended this study to γ-radiation and have discovered another cytotoxic agent with an effect on cellular NAD concentrations.

Treatment of a suspension culture of mouse L1210 leukaemia cells with γ-radiation causes an immediate decrease in cellular NAD, to a minimum after 15 min. This fall is dose-dependent, with a half-maximal effect being produced by 3 krd. The NAD concentration returns to control values after about 8 h.

Neocarzinostatin is a polypeptide antitumour antibiotic of known sequence (Meienhofer *et al.*, 1972) isolated from a variant of *Streptomyces carzinostaticus* (Ishida *et al.*, 1965). When L1210 suspension cells are treated with this agent the NAD concentration falls rapidly. A dose of 5 μg/ml is sufficient to decrease the NAD to 20% of control; recovery of the NAD concentration is very slow and is not complete in 24 h. A 1 h pulse of the drug elicits the full effect.

Poly(ADP-ribose) polymerase is strongly inhibited by 5-methylnicotinamide and the methylxanthines (Davies *et al.*, 1976). The effects of both γ-radiation and neocarzinostatin on cellular NAD can be inhibited by simultaneous treatment with 5-methylnicotinamide or theophylline. If these inhibitors are added after a pulse treatment with the agent, the NAD concentration returns to the control value more rapidly.

Neocarzinostatin is thought to act by single-strand cleavage of DNA (Beerman & Goldberg, 1974). Both alkylating agents and ionizing radiation have major effects on DNA structure which can be demonstrated by alkaline sucrose gradients as single-strand breaks. Cytotoxic agents which do not affect the structural integrity of DNA (to date we have only tested colchicine and 5-fluorodeoxyuridine) have been shown not to affect cellular NAD concentrations. Deoxyribonuclease has also been reported as stimulating the activity of poly(ADP-ribose) polymerase (Janakidevi & Koh, 1974; Miller, 1975).

We propose that damage to DNA results in an increase in the flux through the poly(ADP-ribose) polymerase system which, if acute, is sufficient to lower the cellular concentration of NAD. It is possible that this response is related to DNA repair.

for the two-dimensional gel. The results from the two-dimensional gel confirmed our previous finding (Fig. 1).

Reconstitution of active enzyme (Harding & Beychok, 1974) from isolated subunits (Lowe & Malcolm, 1976) gave 60% of the original activity. On cross-linking α-subunits with either *NN'-o*-phenylenedimaleimide or *NN'-p*-phenylenedimaleimide no activity was observed at all on reconstitution, even after 120min. Since the reconstitution of RNA polymerase proceeds via an $\alpha_2\beta$ intermediate assembly (Ito & Ishihama, 1975):

$$2\alpha + \beta \longrightarrow \alpha_2\beta \xrightarrow[\beta']{} \alpha_2\beta\beta'$$

it follows that RNA polymerase in its normal active conformation must have the α-subunits topologically separated on the surface of the β-subunit and they are linked to each other through the β-subunit.

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Poly(Adenosine Diphosphate Ribose) Glycohydrolase in Germinating Wheat Embryos

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We have previously reported the presence of poly(ADP-ribose) polymerase activity in a crude chromatin preparation from wheat seeds (Whitby & Whish, 1977). Here we report a continuation of this work.

Viable wheat embryos were prepared by a modified method of Johnston & Stern (1957) and stored under vacuum at 4±1°C until use. Viability was shown to be greater than 95%. The embyros were gently homogenized in incubation buffer [0.1M-Tris/HCl (pH8.2)/0.06M-KCl/0.01M-$MgCl_2$/0.004M-KF/2.5mM-dithiothreitol], and gently spun in a bench centrifuge to remove much of the cell debris; the supernatant was lightly respun and the resulting nuclear pellet resuspended in a small volume of incubation buffer.

The poly(ADP-ribose) polymerase activity was determined as previously described (Whitby & Whish, 1977). The results showed a linear incorporation that reached a plateau after approx. 20min. The temperature and pH optima were determined and found to be 26°C and pH8.1 respectively. The incorporation was inhibited by the two inhibitors examined, nicotinamide and 3-aminobenzamide (Purnell & Whish, 1977). These two compounds gave 50% inhibition of poly(ADP-ribose) polymerase at concentrations of 240μM and 1.5μM respectively.

Nicotinamide gave 100% inhibition at a concentration of 20mM, and 3-aminobenzamide gave 100% inhibition at a concentration of 50μM.

By using 3-aminobenzamide, the presence of poly(ADP-ribose) glycohydrolase was investigated in detail.

To 1.0ml of nuclear suspension in incubating buffer was added 70μl of [^{3}H]NAD$^+$ (1mCi/ml; 15Ci/mmol); duplicate blanks were immediately precipitated in 20% (w/v) trichloroacetic acid and the remainder was incubated for a further 20min, at which time 3-aminobenzamide was added to a concentration of 100μM. Inhibition of poly(ADP-ribose) polymerase was complete. Samples were removed during the following 2h incubation.

These results showed that a poly(ADP-ribose)-degrading activity was present in the ungerminated embryo. This poly(ADP-ribose)-degrading activity was further investigated as follows: samples of 100 embryos were placed on filter discs moistened with 1% glucose/0.01% streptomycin as a control, and with 1% glucose/0.01% streptomycin/1mM-cycloheximide; 1mM-cycloheximide has been shown by Obrig *et al.* (1971) to inhibit approx. 90% of protein synthesis. The degree of protein synthesis occurring in the germinating embryos was estimated by following the uptake of [^{3}H]leucine into acid-insoluble material. Five embryos were placed in a small glass tube (50mm×12mm), then 5μl of [^{3}H]leucine was added, followed by 95μl of nutrient solution (1% glucose/0.01% streptomycin, with or without 1mM-cycloheximide) and the tubes placed in an incubator in the dark at 25°C. Uptake was stopped by the addition of ice-cold 20% (w/v) trichloroacetic acid. The embryos were then hand-homogenized in 20% (w/v) trichloroacetic acid and treated as above.

After 23h the cycloheximide-treated embryos had incorporated less than 5% [^{3}H]leucine compared with the embryos not treated with cycloheximide. The incorporation and the presence of 1mM-cycloheximide in the germinating embryos did not affect their poly(ADP-ribose)-degrading activities for at least 20h.

The activity was found to be poly(ADP-ribose) glycohydrolase by showing that the product of poly(ADP-ribose) degradation was ADP-ribose and not 5-phosphoribosyl-5-AMP. The latter would have been expected if degradation had been due to phosphodiesterase activity. It is noteworthy that both poly(ADP-ribose) polymerase and poly(ADP-ribose) glycohydrolase appear to be stable in wheat embryo, since maximal activity remains in nuclei isolated from cells that have been treated with cycloheximide for long periods.

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Effect of Inhibition of Protein Synthesis on the Degradation of Canavanyl-Proteins in *Escherichia coli*

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Escherichia coli possesses a mechanism for the selective degradation of proteins that differ from the normal gene products, e.g. canavanyl-proteins (Pine, 1967; Prouty *et al.*, 1975; Kemshead & Hipkiss, 1974). Although the effects of inhibition of protein synthesis on the degradation of normal proteins in *E. coli* have been quite thoroughly studied (Nath & Koch, 1970; Pine, 1973; Sussman & Gilvart, 1969), effects on the degradation of abnormal proteins (such as canavanyl-proteins) have been subject to scant investigation.

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EFFECT OF POLYAMINES AND Mg^{++} ON POLY(ADP-RIBOSE) SYNTHESIS AND ADP-RIBOSYLATION OF HISTONES IN WHEAT

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SUMMARY: Polyamines in the presence or absence of Mg^{++} have been shown to stimulate 2-4 fold the total synthesis of poly(ADP-ribose) in isolated wheat nuclei. The stimulation by Mg^{++} and polyamines is dose dependent and is an affect on actual synthesis of new chains of polymer since neither Mg^{++} nor polyamines increase the average chain length of the polymer synthesised or inhibit the degradation of the polymer in isolated nuclei. Mg^{++} and polyamine treated nuclei showed an increased ADP-ribosylation of total histones to the same extent as did total polymer synthesised. Furthermore the distribution of poly(ADP-ribose) between histone H1 and the other core histones remained the same in control, Mg^{++} and polyamine treated nuclei.

INTRODUCTION

Mammalian cell nuclei contain a chromatin bound enzyme which catalyses the incorporation of the ADP-ribose moiety of NAD into mono ADP-ribosylated and poly ADP-ribosylated nuclear proteins (1,2). Much information is available from studies employing mammalian cells but the biological significance of poly(ADP-ribose) remains unknown although it has been implicated in DNA repair and replication, transcription, differentiation and cell growth and proliferation (1,2). There is relatively little information available concerning poly(ADP-ribose) in plant tissues (3-6) although plant cell nuclei do possess enzymes for the synthesis (4,5) and degradation (6) of poly(ADP-ribose). In view of the effect of polyamines and Mg^{++} in stimulating poly(ADP-ribose) synthesis in isolated rat liver nuclei (7-9) and also the observation that plant tissues contain fluctuating levels of polyamines (10-14), we describe, in this present report, the effect of polyamines on poly(ADP-ribose) synthesis in isolated wheat nuclei. Since histones are one of the most studied acceptors of poly(ADP-ribose) (1,2) the effect of polyamines on histone ADP-ribosylation in wheat nuclei has also been investigated here in an attempt to gain insight into the possible role of poly(ADP-ribose) as well as to compare the plant and mammalian systems (7,9).

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MATERIALS AND METHODS

Wheat seeds (Triticum aestivus, variety Flanders) were supplied by The West of England Farmers Ltd. Snake venom phosphodiesterase, spermidine and putrescine were purchased from Sigma Chemical Company. Soluene-350 was obtained from Packard. The [adenosine-^{3}H]NAD (1mCi/ml, 20mCi/μmole) was synthesised from [^{3}H] ATP(Radiochemical Centre, Amersham) by the method of Ohtsu and Nishizuka (15).

Isolation of Wheat Embryos: Isolation was performed using the procedure of Johnston and Stern (16) and the embryos were stored in a vacuum dessicator at 4°C until needed.

Isolation of Crude Nuclei: All manipulations were carried out at 4°C. Approximately 0.5 gram viable embryos were homogenised in 15ml 0.1M triethanolamine/HCl pH8.2 using a Potter homogeniser. The supernatant was filtered through two layers of cheesecloth and the residual solid material repeatedly homogenised (approximately 7 times) until no more intact embryos were visible. The filtered homogenate was centrifuged for 2 minutes at 110xg, the supernatant removed and recentrifuged at 2,700xg for 15 minutes. The pellet (crude nuclei) was resuspended in 1ml 10mM mercaptoethanol - 0.1M triethanolamine/HCl pH8.2 and sonicated using an Ultrasonics Rapidas sonicator (setting 50 Watts for 4 x 20 secs using a 3mm probe).

Isolation of Chromatin: Intact seeds were extensively washed with distilled water prior to homogenisation and isolation of chromatin as described by Simon & Becker (17) for the isolation of chromatin from embryos.

Poly(ADP-ribose) Synthesis Assay: 200μl of the sonicated nuclear preparation were incubated with 5μl [^{3}H] NAD in a final volume of 250μl at 26°C. At the appropriate times 3 x 10μl aliquots were taken and spotted on to paper filter discs (Whatman No. 1,2cm) which had been pretreated with 5% TCA in ether for 30 minutes before drying. The discs were then processed for counting by washing 3 times in 5% TCA($^W/_V$) (15 minutes each),once in methanol and finally in ether before drying and counting in 2 ml 0.5% PPO-toluene using a Packard Tri-Carb liquid scintillation spectrometer.

Poly(ADP-ribose) Degradation Assay: Polymer degradation was assayed two ways using either endogenously synthesised poly(ADP-ribose) or exogenously added poly(ADP-ribose) prepared from HeLa cell nuclei which had been incubated with [^{3}H] NAD using the method of Stone et al (18). (1) Endogenous Assay The sonicated nuclear preparation was incubated with [^{3}H]NAD for 10 minutes in the standard assay prior to the addition of nicotinamide (final concentration 100mM) to inhibit further polymer synthesis. Loss of acid precipitable radioactivity was followed with time using the filter disc assay. (2) Exogenous Assay The nuclear preparation was incubated under standard conditions with exogenous [^{3}H] poly(ADP-ribose) and the loss of acid precipitable [^{3}H] polymer determined as a function of time using the filter disc assay.

Average Chain Length Determination: Nuclear preparations were incubated for 10 minutes with [^{3}H] NAD and 100μl aliquots taken and precipitated on ice for 30 minutes with 20% TCA($^W/_V$) (final concentration). The samples were centrifuged at 10,000xg for 5 minutes and washed 3 times with 20% TCA and once with ethanol. The pellets were resuspended in 400μl buffer containing 0.1 unit phosphodiesterase in 50mM Tris/HCl pH8.8, 10mM $MgCl_2$, 20mM glucose-1-phosphate, 4 M urea and incubated at 37°C for 4 hours. TCA (20% final concentration) was added and the sample left on ice for 30 minutes prior to centrifugation at 10,000xg for 5 minutes and extraction of the supernatant 5 times with water saturated ether. Aliquots of the supernatants were applied to PEI-cellulose sheets which were desalted by soaking in anhydrous methanol for 15 minutes prior to development

of the chromatogram as previously described (19). Following chromatography the sheets were dried and cut up into 5mm strips which were then scraped into vials and 200µl 1.6MLiCl added. Two ml of 0.5%PPO - 70% toluene - 30% triton X-100 were added and the samples counted for radioactivity. The average chain length was calculated by dividing the total radioactivity by that of the 5'-AMP radioactivity (2).

Isolation of Histones: Following incubation of nuclei or chromatin with [^{3}H]NAD H_2SO_4 was added (0.4N final concentration) and the samples extracted at 4°C for 1½ hours prior to centrifugation at 10,000xg for 5 minutes. The pellets were re-extracted with 0.4N H_2SO_4 and the combined supernatants precipitated overnight at -20°C with 2-3 volumes of ethanol. The samples were centrifuged and washed 3 times with 20% TCA and twice with ethanol.

Polyacrylamide Gel Electrophoresis: Analysis of the histones was performed using the 6.25 M urea - 0.9N acetic acid gel system of Panyim & Chalkley (20). Histones corresponding to approximately 2 - 4,000 cpm [^{3}H]poly(ADP-ribose) were electrophoresed on duplicate gels at 2mAmp/gel for 2½hours. One gel was stained with Coomassie Blue overnight, destained and scanned using a Chromoscan (Joyce Loebl). The second gel was sliced and the slices dried overnight before incubating with 100µl water and 500µl soluene-350 in capped vials at 50°C for 24 hours. 10ml PPO/triton/toluene scintillant was added and the samples counted for radioactivity.

RESULTS AND DISCUSSION

The polyamines spermine, spermidine and putrescine exhibit a dose dependent effect in increasing the in vitro ADP-ribosylation of nuclei isolated from wheat embryos (Fig. 1A). Mg^{++} also shows a similar but somewhat less effect on ADP-ribosylation (Fig. 1B). The effect of polyamines is seen in the absence (Fig. 2A) or presence (Fig. 2B) of 2mM Mg^{++} which is the optimum Mg^{++} concentration for poly(ADP-ribose) synthesis in this system (Fig. 1B). These results are similar to observations made on the effect of Mg^{++} and polyamines on ADP-ribosylation in rat liver nuclei (7).

There are several possibilities which may account for this observed increase in ADP-ribosylation in the presence of Mg^{++} and polyamines. One possibility is that the degradation of poly(ADP-ribose) in the nuclei might be inhibited by the polyamines and Mg^{++} resulting in the observed increase in poly(ADP-ribose) synthesis. Indeed, poly(ADP-ribose) glycohydrolase, an enzyme which is responsible for the degradation of poly(ADP-ribose) (21-24), is present in wheat nuclei (6). To test this possibility the effect of polyamines and Mg^{++} on poly(ADP-ribose) degradation was investigated. The results are seen in Table 1 and it is clear that polyamines in the presence and absence of Mg^{++}, or Mg^{++} alone, at

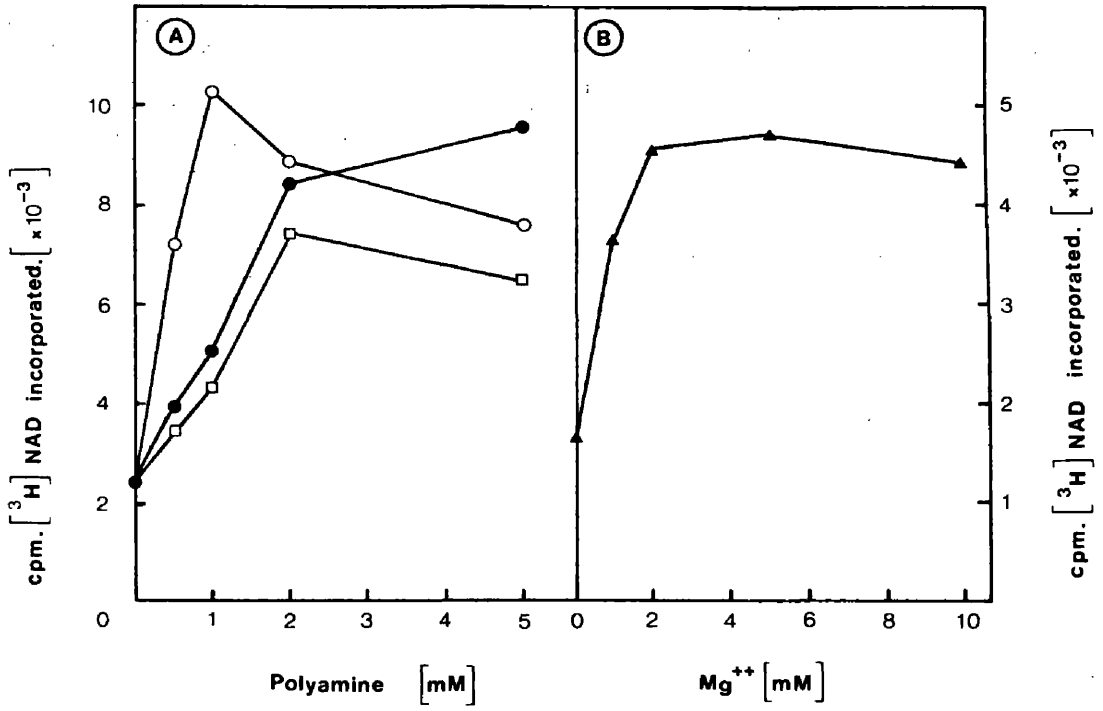

Fig. 1 Effect of polyamine and Mg^{++} concentration on ADP-ribosylation in isolated nuclei. [^{3}H] NAD incorporation into acid insoluble material after incubation for 10 minutes was assayed as in "Methods"., as a function of A) polyamines. —○—, spermine; —●—, spermidine; —□—, putrescine and B) Mg^{++}, —▲—.

concentrations which stimulate poly(ADP-ribose) synthesis (Figs. 1 & 2) do not inhibit the degradation of poly(ADP-ribose when assayed using either exogenously added polymer or *in vitro* synthesised endogenous polymer. In fact there is some stimulation of poly(ADP-ribose) degradation by polyamines (Table 1) which agrees with the observed stimulation of calf thymus poly(ADP-ribose) glycohydrolase by spermidine (22). The slower rate of polymer degradation obtained using exogenous poly(ADP-ribose) (Table 1) is a result of the lower specific radioactivity of this material (data not shown).

A second possibility which may account for the observed increase in poly(ADP-ribose) synthesised in the presence of Mg^{++} and polyamines is that the average chain length of the polymer is increased. The average chain lengths of the poly (ADP-ribose) synthesised in the presence and absence of Mg^{++} and polyamines have been determined and the results are given in Table 1. The values are all in very close agreement indicating that the Mg^{++} and polyamines cause an

Table 1

Effect of polyamines and Mg^{++} on the average chain length and degradation of poly (ADP-ribose) in isolated wheat nuclei

Addition	Poly (ADP-ribose) degradation* (% polymer degraded / min)		Average Chain Length
	Exogenous Assay	Endogenous Assay	
Control	0.66	3.45	2.51
Mg^{++} (2mM)	0.64	1.58	3.22
Spermine (1mM)	1.07	4.6	2.81
Spermidine (1mM)	1.02	6.46	3.10
Putrescine (1mM)	0.67	4.37	3.32
Spermine (1mM) + Mg^{++} (2mM)	0.87	4.78	2.88
Spermidine (1mM) + Mg^{++} (2mM)	0.84	6.08	3.14
Putrescine (1mM) + Mg^{++} (2mM)	0.91	3.87	3.41

The assay was performed as described in "Methods"

* 100% poly (ADP-ribose) = 4,000 cpm/10µl aliquot in both cases

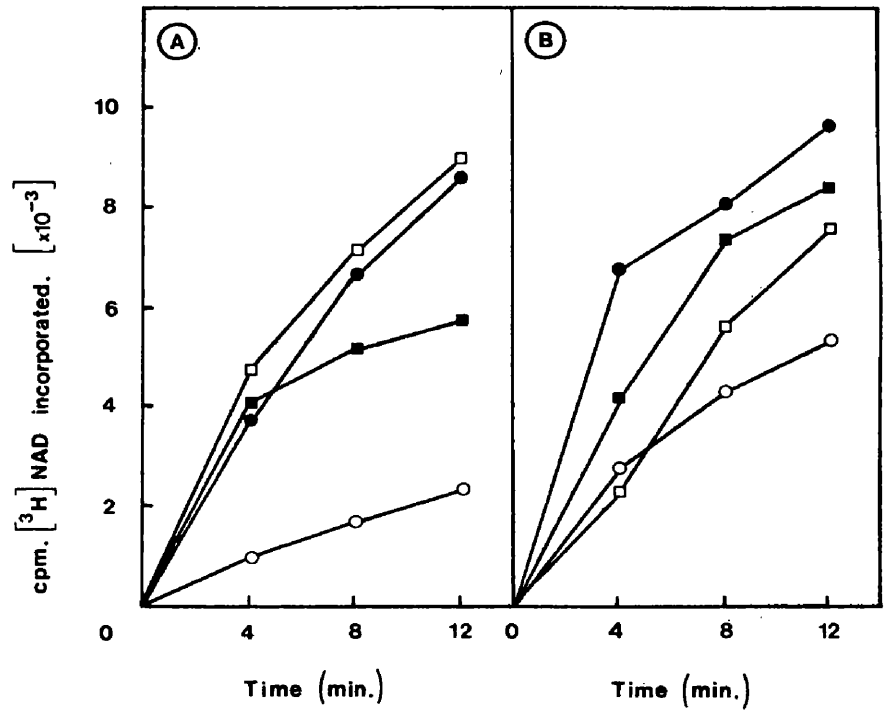

Fig.2 Effect of 1mM polyamines on the rate of ADP-ribosylation in isolated nuclei. [^{3}H] NAD incorporation into acid-insoluble material was assayed as described in "Methods" A) in the absence of any Mg^{++}, B) in the presence of 2mM Mg^{++}. —O—, control (no polyamine); —●—, spermine; —□—, spermidine; —■—, putrescine.

increase in the number of polymer chains synthesised and have little, if any, effect on the average chain length of poly(ADP-ribose).

The possibility exists that the new poly(ADP-ribose) chains synthesised in the presence of Mg^{++} or polyamines may be preferentially associated with a specific nuclear protein fraction (e.g. histones, non-histones). This seems to be the case in rat liver nuclei where it has been shown that ADP-ribosylation occurs mainly in non-histones in the presence of 1mM spermine while in the presence of Mg^{++} ADP-ribosylation occurs mainly in the histones (7). Other workers have also observed an increased ADP-ribosylation of non-histone proteins in the presence of both Mg^{++} and spermine (8,9). To investigate whether this same effect of Mg^{++} and polyamines occurs in wheat we have incubated nuclei with [^{3}H] NAD in the presence and absence of Mg^{++} and polyamines and analysed the extracted histones by polyacrylamide gel electrophoresis. The profiles of the ADP-ribosylated histones synthesised in chromatin, (Fig.3A) and nuclei (Fig. 3B) incubated in the absence of Mg^{++} and polyamines indicate that histone H1 is

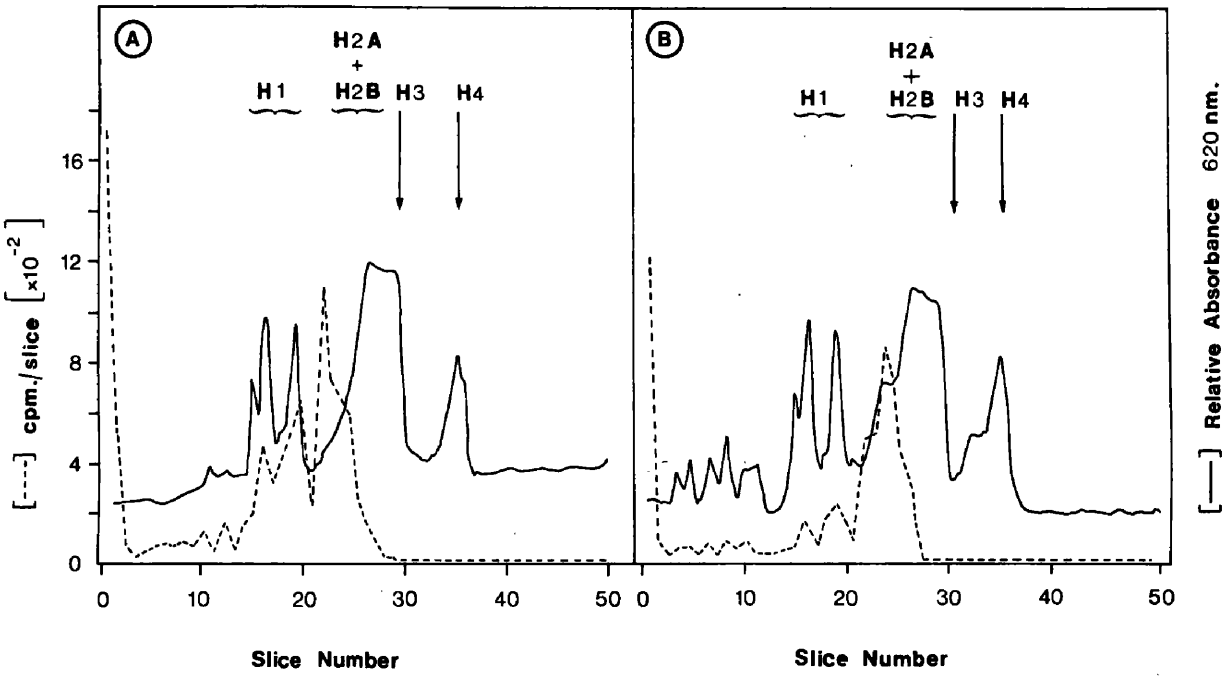

Fig. 3 Distribution of [^{3}H] poly (ADP-ribose) on acetic acid-urea polyacrylamide gels. Electrophoretic analysis was performed using histones extracted from A) chromatin and B) nuclei that were incubated with [^{3}H] NAD for 10 minutes at 26°C as described in "Methods".

ADP-ribosylated, and maximum incorporation is seen in histones H2A/H2B, although in the case of the latter the ADP-ribose profile does not coincide with the protein profile. This is not too surprising since heavily ADP-ribosylated histone H1 electrophoreses slower than unmodified H1 (8,9,25-28). There is no ADP-ribosylation of H4 and no or very little modification of H3 (Fig. 3A,B). The actual histone profiles from wheat differ from those of mammalian cells in respect to the mobility of H1, H2A and H2B, and the profiles presented here are in agreement with previously published histone analyses from wheat and other plant sources (29).

Similar gel analyses were performed on histones extracted from nuclei incubated with [^{3}H] NAD in the presence and absence of Mg^{++} and polyamines. The results are summarised in Table 2 and it can be seen that the stimulation of total poly(ADP-ribose) synthesis is accompanied by a similar increase in the total histone associated poly(ADP-ribose) for Mg^{++} and polyamines. These results differ from those obtained in rat liver (7) and show that in wheat nuclei there is no preferential ADP-ribosylation of histones caused by Mg^{++} or polyamines. It is also seen that the distribution of ADP-ribose between H1 and the other

Table 2

Distribution of {^{3}H} poly (ADP-ribose) in Histone Fraction

Addition	{^{3}H} Total Acid Insoluble poly (ADP-ribose) cpm	Total Histone cpm	Total Histone % of total	Histone H1 cpm* %	Histone H2A/H2B cpm* %
Control	76,562	10,420	13.6	21	79
Mg^{++} (2mM)	200,581	37,660	18.8	26	74
Spermine (1mM)	209,156	45,360	21.7	20	80
Spermidine (1mM)	192,762	32,890	17.1	19	81
Putrescine (1mM)	136,990	26,330	19.2	24	76

{^{3}H} ADP-ribosylated histones were electrophoresed on acetic acid-urea polyacrylamide gels and processed as described in "Methods"

* determined from gel analyses.

histones is not greatly affected by Mg^{++} or polyamines. This is again different to the situation in rat liver where it has been shown that 1mM spermine causes no change in the extent of ADP-ribosylation of total histones although a redistribution of the ADP-ribose from the core histones to H1 was observed (8,9).

Whether such discrepancies seen between the wheat and rat liver system reflect inherent differences in the ADP-ribosylation machinery or whether they arise as a result of the unique features of the wheat acceptor proteins (H1, H2A and H2B) remains to be seen. However, in view of the observed ADP-ribosylation of these histones in isolated wheat nuclei and the stimulation by polyamines it may well be that ADP-ribosylation of histones in plant cells is involved in extending or condensing chromatin as has been postulated for mammalian cells (8,9,26-28). Whether such a mechanism occurs in vivo is unknown at present and further methodology needs to be developed in order to analyse in vivo levels of ADP-ribosylated proteins. We are currently proceeding in this direction.

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Adenosine diphosphate ribosylation of proteins in rat pancreas

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ADP-ribosylation of nuclear proteins is now known to occur in the nuclei of all organisms tested to date. The poly(ADP-ribose) system has been studied in great detail in a large number of systems *in vitro* and to a much lesser extent *in vivo* [for a review see Hilz & Stone (1976)]. Mono(ADP-ribosylation) is also known to occur and appears to play quite a different role from poly(ADP-ribose) (Whish & Surowy, 1980). However, the function of either of these protein modifications in the nucleus is still unknown, though poly(ADP-ribose) has been implicated in DNA synthesis (Hayaishi & Ueda, 1977), DNA repair (Davies *et al.*, 1977) and cellular differentiation (Young & Sweeney, 1979). In an attempt to determine the function of nuclear protein ADP-ribosylation, a model system was chosen that might usefully distinguish between the various functions ascribed to ADP-ribosylation of nuclear proteins. For this reason and because it is extremely well characterized biochemically, the rat pancreas was chosen as such a model.

The pancreas was excised from male Sprague–Dawley rats (300–350g) and washed in Krebs–Ringer phosphate medium. Each pancreas was then preconditioned in 5ml of the above medium with shaking at 37°C for 10min in an atmosphere of CO_2/O_2 (1:19). The pancreas was removed and placed in 3ml of fresh medium which contained 120μCi of [^{3}H]adenosine with a specific radioactivity of 20Ci/mmol. Incubation was continued for a further 5h. At this time the pancreas was rinsed in ice-cold saline and homogenized in 10ml of 80% (v/v) ethanol containing 0.5M-acetic acid. The suspension was then centrifuged at 40000g for 10min and the supernatant discarded. The pellet was resuspended in 10ml of 80% (v/v) ethanol and again centrifuged. The pellet was washed in this manner until there was less than 200c.p.m./ml of supernatant. The pellet was suspended in 2ml of 8M-urea and to this was added 2ml of 100% (w/v) CsCl in 0.05M-sodium acetate, pH5.0. The clear solution was then centrifuged to density equilibrium at 150000g in a Beckman L5-50 ultracentrifuge. The centrifugation usually took at least 60h, after which time the completed density gradient was fractionated and the acid-insoluble radioactivity determined in each fraction. Under the conditions of centrifugation there was complete separation of DNA and RNA, which both sedimented to the bottom of the tube, and [^{3}H]adenosine-containing proteins, which banded at the top of the tube. The proteins were precipitated from the CsCl/urea with a final concentration of 20% (w/v) trichloroacetic acid, collected by centrifugation at 40000g and washed twice with 80% (v/v) ethanol. The resulting pellet was then resuspended in 0.05M-Tris/HCl, pH7.5. This suspension contained 95% of the total acid-insoluble radioactivity originally present in the CsCl/urea fraction. The acid-insoluble [^{3}H]adenosine was completely resistant to deoxyribonuclease I, and moreover the preparation contained no detectable deoxyadenosine on complete digestion with snake-venom phosphodiesterase and alkaline phosphatase, thus proving that DNA was not present. About 15% of the radioactivity in the preparation was rendered acid-soluble with ribonuclease and/or spleen phosphodiesterase, indicating that some RNA was present. Complete solubilization of the acid-insoluble material occurred on digestion with snake-venom phosphodiesterase. The resistance of the acid-insoluble radioactivity to deoxyribonuclease I, ribonuclease and spleen phosphodiesterase and its complete solubilization by snake-venom phosphodiesterase indicates that the [^{3}H]adenosine has been incorporated into ADP-ribose or poly(ADP-ribose). Unambiguous proof of this can be obtained by alkali hydrolysis of the acid-insoluble material and subsequent analysis of the products. DNA is unaffected by overnight treatment with dilute alkali, RNA is hydrolysed to 3′-AMP, ADP-ribose to 5′-AMP (Goebel *et al.*, 1977), and poly(ADP-ribose) is removed from protein but is otherwise unaffected by the base. Thus the acid-insoluble material was treated with 0.1M-NaOH at 37°C overnight, neutralized with HCl, and the products were then analysed by t.l.c. By a variety of standard procedures it was shown that the acid-insoluble material, when completely hydrolysed by alkali, gave 15% 3′-AMP (RNA), 75% 5′-AMP (ADP-ribose) and 10% poly(ADP-ribose). The identity of the latter compound was proved by showing that, on digestion with snake-venom phosphodiesterase, the product was phosphoribosyl-AMP, which is unique to poly(ADP-ribose). 5′-AMP might also rise from adenylated proteins, but for these to be digested by snake-venom phosphodiesterase requires that they are linked to protein via a second phosphate group. We are not aware, however, of any ADP-proteins with the properties that we describe. Moreover t.l.c. analysis shows that the transient intermediate of alkali hydrolysis is not ADP but ADP-ribose.

It can be concluded therefore that the pancreas will incorporate [^{3}H]adenosine into ADO-ribosylated protein. Most of the incorporation is into monomeric ADP-ribose, but, although only 10% of the label is in poly(ADP-ribose), this does represent about 5×10^5c.p.m. per pancreas.

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An inhibitor of poly(adenosine diphosphate ribose) glycohydrolase found *in vivo* in germinating wheat embryos

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Poly(ADP-ribose) is a nuclear polymer bound covalently to protein which is formed by poly(ADP-ribose) synthetase from the coenzyme NAD^+. The polymer is degraded by a second enzyme, poly(ADP-ribose) glycohydrolase, to form ADP-ribose [for a review see Hilz & Stone (1976)].

Both of these enzymes and the protein–polymer complex are tightly bound to chromatin. A variety of functions has been ascribed to the poly(ADP-ribose) system, such as control of DNA synthesis, involvement in DNA repair and in cellular differentiation. The actual biological role of the polymer is unknown.

Previous reports from this laboratory have shown the existence of poly(ADP-ribose) synthetase and poly(ADP-ribose) glycohydrolase in wheat embryos (Whitby & Whish 1977,

1978). Our work has also shown that, on imbibition of water, the wheat-embryo glycohydrolase activity decreased by 95% over a period of 6h. However, the reason for this decrease in enzyme activity was unknown. It is possible that the enzyme activity might be related to the physical state of the chromatin, that is, the more condensed the chromatin, the lower the glycohydrolase activity becomes, simply because the enzyme is less accessible. Chromatin may be solubilized, and thus decondensed, experimentally by using high salt concentrations. Therefore an increase in enzyme activity should be seen if condensed chromatin is treated with salt and then assayed. To explore this possibility, the effect of NaCl on the activity of poly(ADP-ribose) glycohydrolase was determined in nuclei from both 2h- and 6h-imbibed embryos. In the former, where glycohydrolase activity is maximal in buffer, some 50% further increase in activity was seen with an optimal NaCl concentration of 0.25M. However, no salt stimulation of enzyme activity occurred in the nuclei from 6h-imbibed embryos, where the initial activity was minimal. Thus the low enzyme activity seen in the latter case is not simply due to the chromatin-condensation effects discussed above.

An alternative explanation for the large decrease in glycohydrolase activity is that, on imbibition of water, the embryo produces a poly(ADP-ribose) glycohydrolase inhibitor that is expressed fully at 6h. To investigate this possibility experiments were carried out whereby nuclear extracts of 6h-imbibed embryos were found to cause almost complete inhibition of the glycohydrolase in nuclei that were known to contain large amounts of enzyme. Thus it appears that the wheat embryo does indeed produce an effective inhibitor of poly(ADP-ribose) glycohydrolase. This is an important observation because to date no effective physiological inhibitor of the enzyme has been described.

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Action of dithiothreitol and mersalyl on trypsin followed by naphthylamidase assay and active-site titration

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Trypsin has been shown to possess a significant disulphide bond that controls the conformation of the active centre; thiols form intermolecular complexes through this disulphide bond with consequent inhibition of trypsin (Steven & Podrazký, 1978, 1979). We now demonstrate the direct involvement of this disulphide bond in complex-formation by following the reaction with active-site titration (Chase & Shaw, 1967).

In all experiments the trypsin activity was measured by means of the fluorimetric naphthylamidase assay (MacDonald *et al.*, 1966), and the results are expressed as percentages of the control trypsin used for each series of experiments. The data in Fig. 1 show the initial inhibition of trypsin by dithiothreitol followed by re-activation in the presence of incremental additions of cystine or mersalyl. In Fig. 1(i) 2μg of trypsin plus 100μM-dithiothreitol caused an initial inhibition in all tubes represented by the fall from *A* to *D*, due to rupture of the disulphide bond in trypsin. The addition of cystine resulted in progressive re-activation of trypsin (curve *D–E–C*) by disulphide exchange (Steven & Podrazký, 1978, 1979). In Fig. 1(ii) the disulphide bond of trypsin (1μg) is protected from attack by the added 100μM-dithiothreitol by incremental additions of the organomercurial mersalyl [Evans Medical; 10% (w/v) solution]. This plot (*D–F–C*) demonstrates that the function of the mersalyl is to bind free thiols and displace thiols from trypsin–thiol complexes. In Fig. 1(iii) the events in Fig. 1(i) and 1(ii) have been combined. We first incubated 2μg of trypsin with dithiothreitol (position *B*) and then added incremental additions of

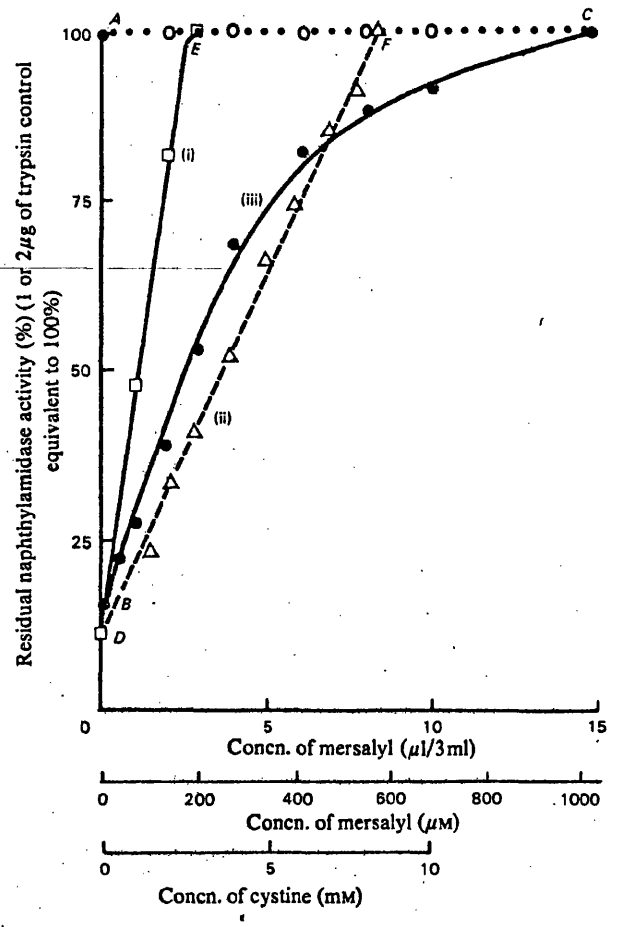

Fig. 1. *Re-activation of trypsin*

Experimental details are indicated in the text. Point *A* represents initial trypsin activity, the dotted line *A–C* represents trypsin plus mersalyl (○). Curve (i) 2μg of trypsin plus 100μM-dithiothreitol followed by cystine produced re-activation along curve *D–E–C* (□). Curve (ii) 100μM-dithiothreitol plus mersalyl followed by addition of 1μg of trypsin resulted in the plot *D–F–C* (△) demonstrating mersalyl protection of trypsin against dithiothreitol. Curve (iii) 2μg of trypsin plus 100μM-dithiothreitol followed by mersalyl demonstrated re-activation of trypsin along curve *B–C* (●).