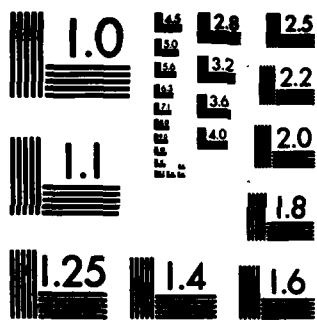


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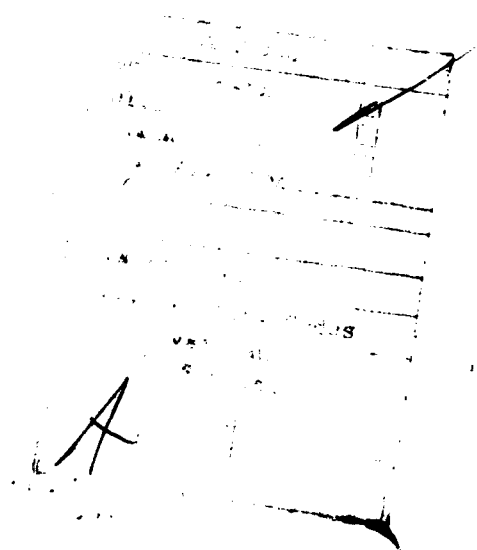
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REGULATION OF CHROMATIN FUNCTION BY POLYADENOSINE DIPHOSPHORIBOSYLATION

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[XIIth Steenbock-Lilly Symposium. June 7-11, MADISON, 1982]

INTRODUCTION. It was discovered in 1965-67 and confirmed 10 years later that pharmacological doses of nicotinamide induce a transient increase in the biosynthesis of hepatic tryptophane pyrrolase, tyrosine transaminase (1, 2) and cardiac ornithine decarboxylase (3, 4). No nicotinamide related metabolic pathway other than the polymerization reaction leading from NAD to the nuclear homopolymer poly(ADP-R) could be implicated in the regulation of the process of enzyme induction. It was concluded that inhibition of the polymerization pathway by large doses of nicotinamide may have a modifying (de-repressing) effect on rates of selective protein synthesis. The most probable site of this type of regulation appeared to be at the transcription level because the selective nuclear localization of poly(ADP-R) confined covalent protein modification to chromatin. It was therefore postulated that a detailed study of covalent modification of nuclear proteins by poly(ADP-R) may lead to the recognition of new mechanisms of chromatin regulation by this natural product. Since our early observation (1) some aspects of the enzymology and primary structure of poly(ADP-R) were described (5). However, neither the macromolecular properties of the homopolymer nor its physiological function have been adequately clarified.

The present report is confined to a description of ongoing investigations in this laboratory concerned with: a) macromolecular properties of the polydisperse homopolymer, identifying it as a nucleic acid exhibiting secondary structure, b) the enzymatic mechanisms of the synthesis and degradation of the homopolymer in intact cell nuclei of permeabilized cells, representing physiological conditions, c) a physiologically meaningful participation of poly(ADP-R) in the regulation of RNA synthesis in cardiocyte nuclei and d) the role of poly-ADP-ribosylation in cancer promotion and the prevention of carcinogenesis by an inhibitor of the synthetase in human fibroblasts.

MATERIALS AND METHODS. Large scale isolation of pure polydisperse protein free poly(ADP-R) was carried out by a recently developed affinity chromatography technique (6, 7) and chain lengths determined as reported earlier (8). Synthetase and glycohydrolase assays and isolation of nuclei were performed as

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published previously (9, 10) and gel electrophoretic separation of protein-polymer adducts by recently developed techniques (11, 12). Culturing of 9L-Rat Brain Gliosarcoma cells (13) and their permeabilization (14) as well as conditions of incubation and treatment are described elsewhere (11). In vitro carcinogenesis with human fibroblasts was tested as has been reported (15).

RESULTS AND DISCUSSION.

a) Macromolecular properties of polydispers poly(ADP-R).

In contrast to covalent modification of proteins by phosphorylation, acetylation, adenylation, etc., where the modifying groups are relatively small molecules, the homopolymer of ADP-R is a macromolecule itself, extending to polymers of at least 40 to 70 ADP-R units long (5, 8, 10). We assumed that the macromolecular nature of the homopolymer could play an essential function in either facilitating or inhibiting protein-protein or protein-nucleic acid associations (16). The possibly specific promoter or inhibitory role of poly(ADP-R) in macromolecular interactions in chromatin, serving a biological cross linking function, would appear improbable if poly(ADP-R) chains resembled the random structure of polysaccharides. Apart from the primary structure of the homopolymer consisting of repeat α -glycosidic ribose-ribose and pyrophosphate bonds (17-20), no information exists that deals with macromolecular structural aspects of poly(ADP-R). With the aid of a newly developed preparative method (6) sufficient quantity of highly purified polydisperse poly(ADP-R) became available for the correlation of spectral properties of oligomers with their chain length (6).

When A_{280}/A_{260} ratio and hypochromicity of oligomers is plotted against the length of oligomers, separated by molecular filtration on Sephadex G-50, a characteristic correlation is obtained as shown in Fig. 1.

It is evident that at a chain length of 9 ADP-R units a transition occurs, consistent with the appearance of a secondary structure that is chain length dependent. The developing solvent in this experiment was 1 M NaCl buffered with 100 mM Tris Cl to pH 7.0 (molecular filtration, bottom curve Fig. 1).

With long chain polymers ($n = 35 - 45$) the concentration of the solvent was varied from 0 to 1000 mM phosphate (pH 7.0). An ionic strength dependent increase in A_{280}/A_{260} was observed as illustrated in Fig. 2.

A temperature dependence of A_{280}/A_{260} was also observed that was largest for long chain polymers (not shown). This chain length and ionic strength correlated spectral behavior of poly(ADP-R) strongly suggested a secondary structure of the homopolymer, a conclusion further confirmed by circular dichroism analyses, a technique that has been particularly suitable for conformational analyses of nucleic acids (21, 22, 23).

Fig. 1.

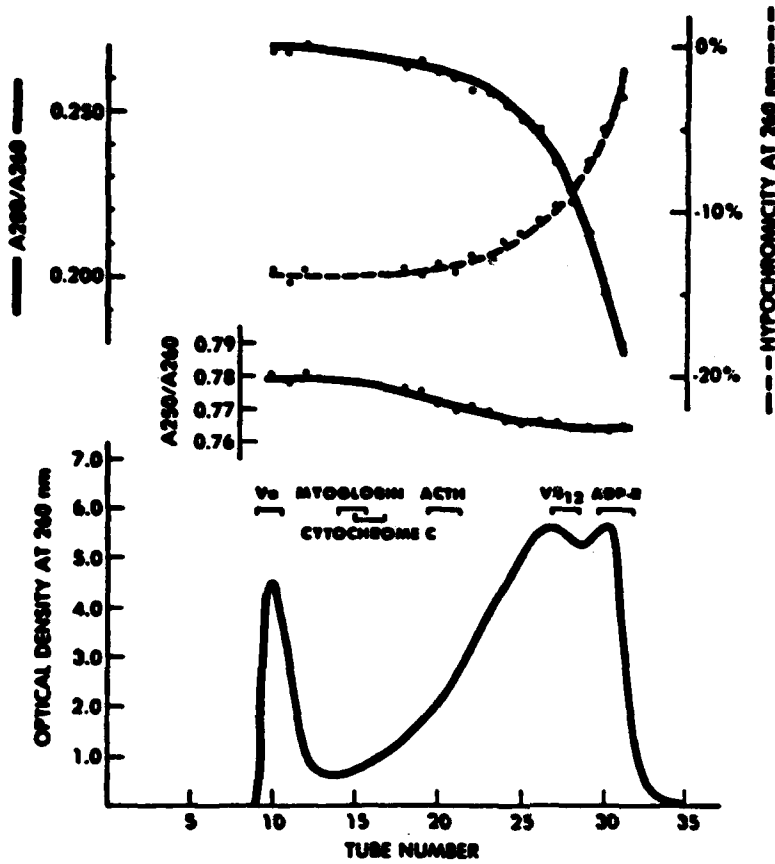
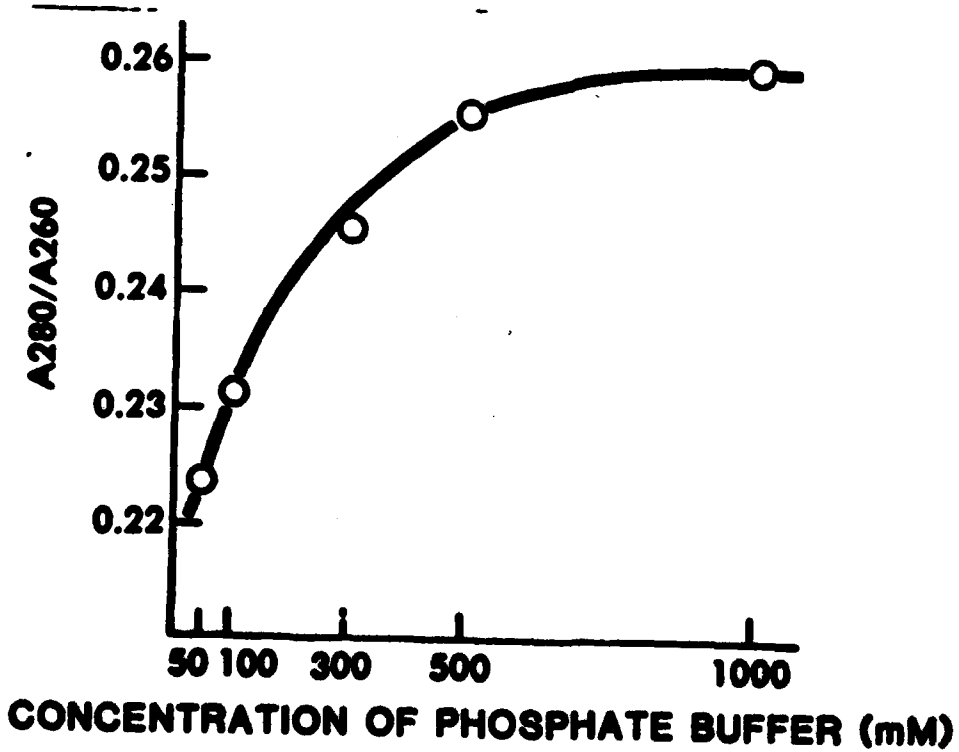


Fig. 2.



As illustrated in Fig. 3 characteristic θ values of long chain ($n = 45$) polymers exhibited a marked temperature dependence. At 5° two positive bands (272.5 and 205 nm) and one large negative band (at 249.5 nm) were apparent with a shoulder at 222 to 225 nm. However, at 72°, both large positive and negative bands disappeared and were replaced by a new negative band at 267.5 nm with isosbestic points at 228 and 259 nm. A clearly recognizable red shift occurred between 5° and 72° and a melting curve was obtained by plotting θ at 249.5 nm against temperature (inset). A comparison of CD spectra of long ($n = 45$), medium ($n = 15 - 20$) and short ($n = 4 - 9$) oligomers at three temperatures are summarized in Fig. 4.

Largest effects were seen with long chain polymers. Between 5° and 25° the red shift with oligomers of medium chain length was readily discernible because only the position of the peak of θ but not its magnitude varied. In case of short oligomers no red shift was seen and at 72° the differences between CD spectra of varying chain length completely disappeared.

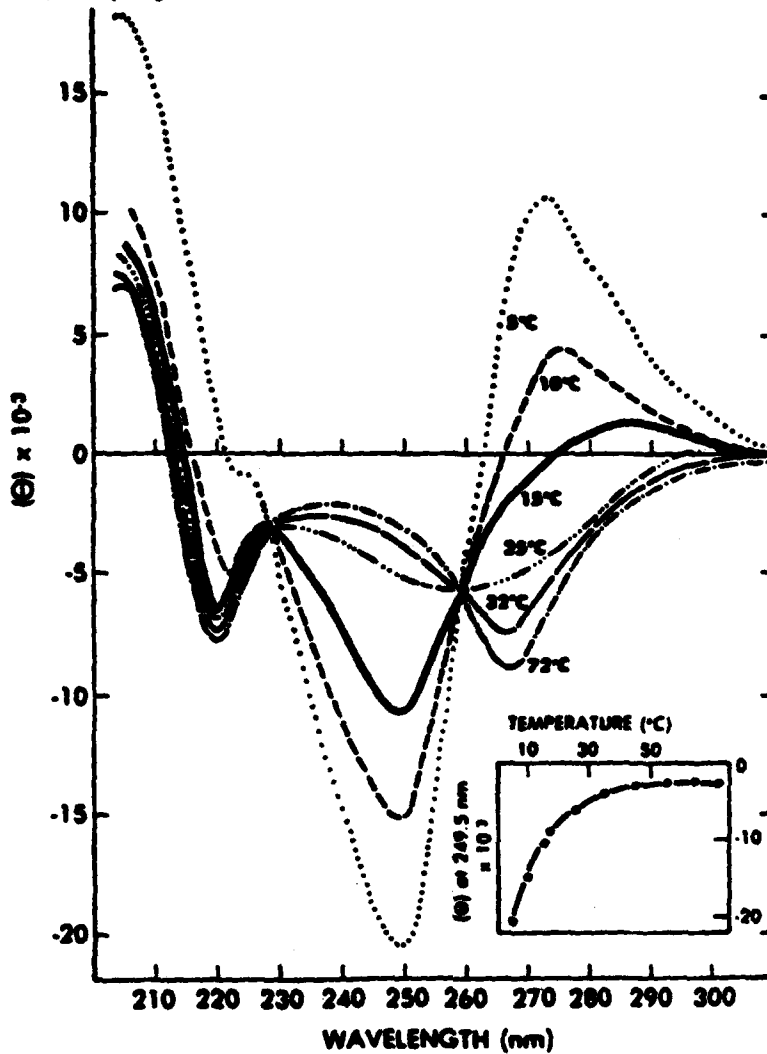
Although precise interpretation of these results depends on further studies (e.g. X-ray diffraction) that are presently pursued, it is evident that poly-(ADP-R) exhibits characteristic structural parameters reminiscent of nucleic acids, and this homopolymer can justifiably be defined as the third nucleic acid.

b) Enzymatic mechanism of the synthesis and degradation of poly(ADP-R) in intact cell nuclei.

Although the enzyme which apparently catalyzes the synthesis of poly(ADP-R) was first isolated in Hayaishi's laboratory (cf. 5) and identified as a protein of a molecular mass of 110-120 kd, there is considerable uncertainty related to its catalytic mechanism as well as its integration with intact chromatin. In the purified form of the enzyme two distinct catalytic functions are retained; one is an NAD glycohydrolase activity, releasing free ADP-R and NA, the second, is the polymerase activity that utilizes the enzyme as a covalently binding protein template. Auto-poly ADP-ribosylation of the pure enzyme results in an inhibition of the catalytic activity of the enzyme and no transfer of ADP-R from the enzyme bound polymer to other protein acceptors takes place (24), a situation which is in sharp contrast to conditions measured in vivo, where a large variety of mostly non histone proteins are poly ADP-ribosylated (8, 9, 10, 11, 12). We have found that ADP-R readily forms Schiff bases with a variety of proteins (25) and it was shown that these adducts can serve as templates for polymer elongation (26). It appears that the NAD glycohydrolase activity of the polymerase by releasing ADP-R can serve as an initiator, forming protein-ADP-R adducts (25) to which polymer chains can be added, comprising a reasonable

alytic process of polymerization.

Fig. 3.



We have approached this problem by determining polyadenosine diphosphoribose formation in nuclei of permeabilized cells representing a physiological condition (11) and compared the experimentally obtained chain length distribution at equilibrium with that calculated according to Florey (27). Assuming a linear relationship between chain length and fraction number (8) with W_n max occurring at $n = 70$ we plotted $W_n/W_n \text{ max}$ against chain length (n) and calculated a Poisson distribution according to: $W_n/W_n \text{ max} \approx (v/n)^n \exp. (n - v)$ with $v = 70$.

As shown in Fig. 5, there is coincidence at longer chain length between experimental values (dots) and the calculated distribution (solid line), but a discrepancy at shorter chain length.

The most plausible cause for this departure is in a collection of initiating sites differing in the rate at which chains are commenced, i.e. ADP-R

Fig. 4..

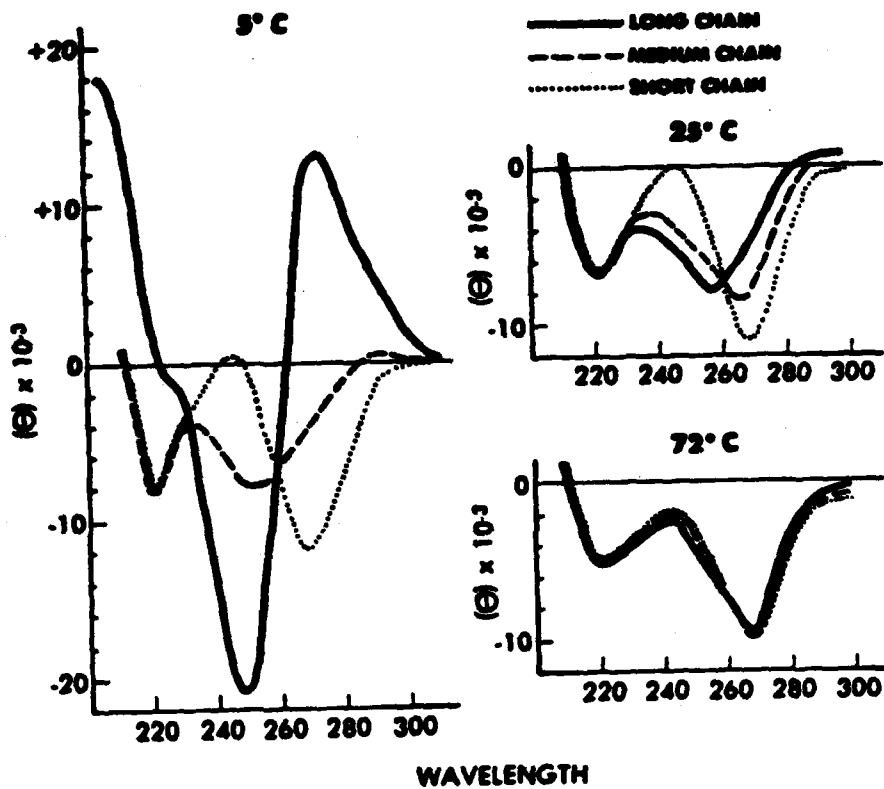
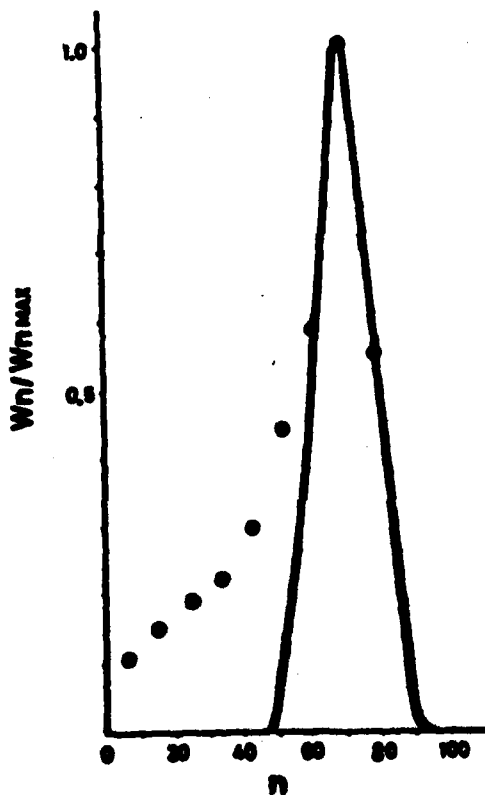


Fig. 5.



protein adducts are formed. Once chains are initiated and surpass some minimum size their growth rate should be invariant and no longer reflect their point of origin. It follows that the most critical regulation is at the nucleophilic protein sites of ADP-ribosylation and recognizing this fact it is now feasible to search for specific nuclear protein acceptors relevant to the physiological function of poly ADP-ribosylations.

The rates of degradation of intranuclearly formed polymers exhibit an inverse relationship to the concentration of the intranuclear polymer, as shown in Table I.

TABLE I.

The influence of intranuclear concentration of poly(ADP-R) formed from 50 or 500 μ M NAD on the decay of poly(ADP-R).

Externally added NAD	Nuclear concentration of poly(ADP-R)*	$t_{1/2}$ poly (ADP-R) decay
50 μ M	0.6 μ M	3 min.
500 μ M	13.0 μ M	18 min.

* as μ M ADP-R.

Cells were incubated with 50 or 500 μ M NAD for 10 minutes, then the synthetase reaction was stopped by a specific inhibitor (benzamide) and $t_{1/2}$ of decay determined.

This unexpected intranuclear kinetics indicates that at high NAD levels where the polymer content is high the rate of polymer degradation is slow. This type of kinetic regulation has important metabolic consequences since poly(ADP-R) is in the direct catabolic path of NAD turnover (28); thus the cellular regulation of NAD content depends on poly(ADP-R) glycohydrolase activity. The probable enzymatic basis of the regulatory phenomenon is the high sensitivity of poly(ADP-R) glycohydrolase to product inhibition. It also follows that at high concentration of cellular NAD its catabolism via poly(ADP-R) is slow, whereas when NAD concentration is decreased as in various pathophysiological conditions (e.g. precancerous state, cf. 10) catabolic rates of both NAD and the polymer are increased. The cellular physiological consequences of these kinetic phenomena, indicating an intimate coupling of metabolic and epigenetic processes, represent an as yet unexplored new field.

c. Participation of poly(ADP-R) in the regulation of RNA synthesis in cardiocyte nuclei.

Cardiac enlargement induced by stress is probably mediated by developmental hormones and we have shown (29-33) that poly ADP-ribosylation is

characteristically diminished by thyroid and steroid hormones in vivo, an effect also reproducible in vitro with isolated nuclei at hormone concentrations of $10^{-7}M$ (31). The correlation between triiodothyronine treatment induced cardiac enlargement, poly(ADP-R) and RNA synthesis is shown in Table II.

TABLE II.

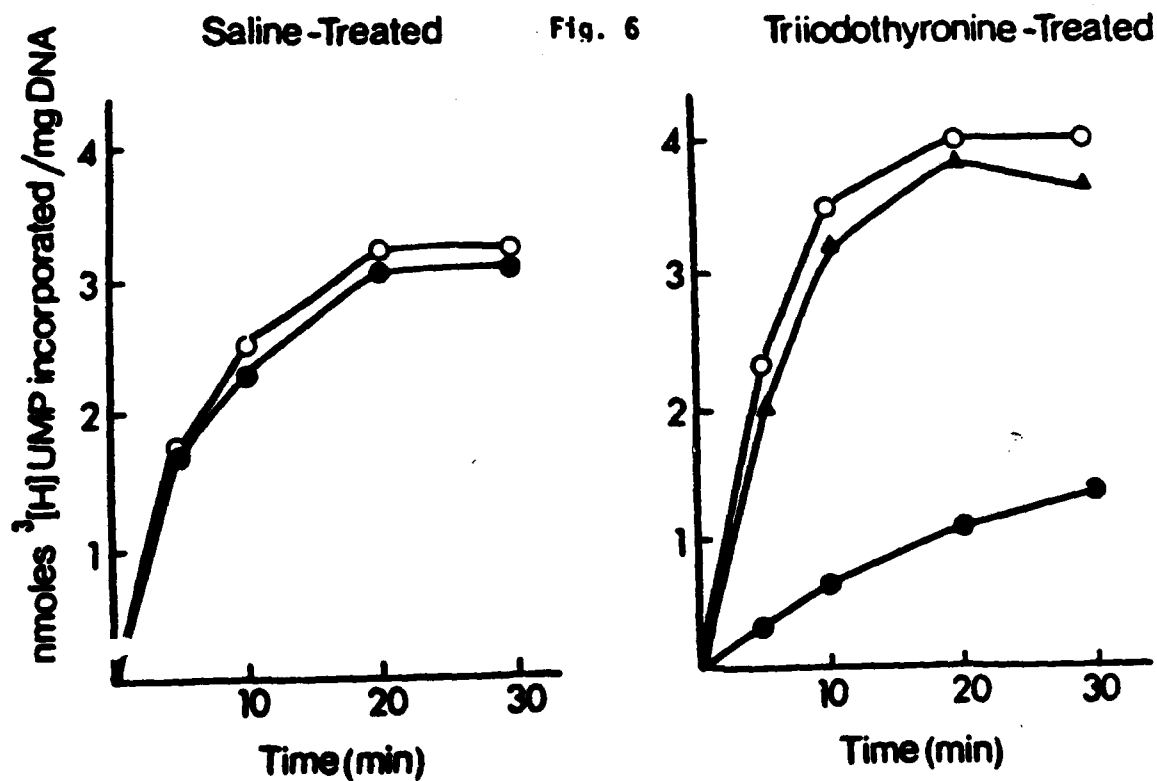
The effect of triiodothyronine administration on heart weight and poly(ADP-R) and RNA polymerase activities of cardiocyte nuclei.

No.	Experimental Conditions	Heart Weight (in g)	Cardiocyte nuclear	
			poly(ADP-R) polymerase, n mol ADP-R/ mg DNA in 10 min.	RNA polymerase, p mol UMP/mg DNA in 10 min.
1.	Normal control (saline treated n = 24)	0.28 ± 0.02	195 ± 10	400 ± 10
2.	T ₃ treated* (n = 24)	0.41 ± 0.02	54 ± 4	700 ± 20

* 30 µg/100 g i.p. for 4 days.

The complex mechanism of these phenomena is illustrated in Fig. 6, where the effects of in vitro poly ADP-ribosylation in isolated cardiocyte nuclei on RNA synthesis in vitro was determined. Cardiocyte nuclei isolated from normal rats, if poly ADP-ribosylated (by preincubation with 0.5 mM NAD + Mg²⁺ for 30 minutes at 25°) show no modification of RNA synthesis (left half of Fig. 6; —○—○= control; —●—● after poly ADP-ribosylation). However, when the same experiment is performed with cardiocyte nuclei isolated from rats treated for 4 days with 30 µg/100 g triiodothyronine, poly ADP-ribosylation in vitro profoundly inhibits RNA synthesis (right part of Fig. 6, —○—○ = controls; —●—● after poly ADP-ribosylation). To demonstrate that not NAD but poly(ADP-R) was the inhibition producing component, in a third experiment the poly(ADP-R) polymerase was inhibited by 20 mM nicotinamide (that itself has no effect in this system) and there was no inhibition when nuclei were preincubated with NAD + NA (Fig. 6, right side —▲—▲ preincubation with 0.5 mM NAD + 20 mM NA). These results cannot be explained by a direct inhibition of RNA synthesis by poly ADP-ribosylation of RNA polymerase enzymes even though poly ADP-ribosylation of RNA polymerase has been demonstrated (34). It is well known that developmental hormones augment selective protein synthesis (35) and the large increase of cardiac tissue demonstrates this effect. That inhibitory effect of poly ADP-ribosylation on RNA synthesis in vitro occurs only after T₃ stimulated

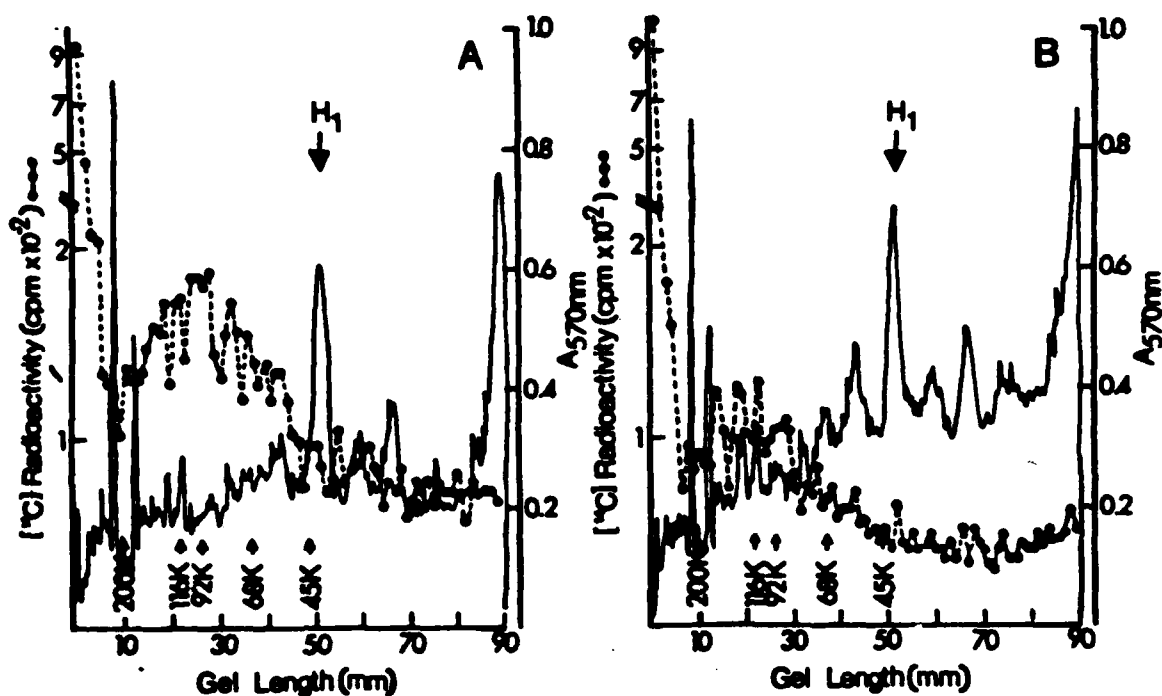
hypertrophy suggests that proteins that have been induced by T_3 treatment can exert an inhibitory effect on RNA polymerases only when poly ADP-ribosylated. Poly ADP-ribosylation apparently serves as a coupling or cross linking agent between inducible regulatory non histone proteins and their sites of action presumably at the DNA template level. In terms of physiology, the reversal of the phenomena shown in Fig. 6 may represent the return to normal resting state after the poly (ADP-R) synthetase inhibitory (hormonal) factors diminished to a low concentration existing in the non stimulated state.



It follows that poly(ADP-R) can serve as a marker for the identification of proteins that may be deterministic in the regulation of selective transcription processes. This type of mechanism predicts some degree of selectivity in the nature of poly(ADP-R) accepting nuclear proteins and this has been observed experimentally (12). Fig. 7 illustrates a one dimensional separation by gel electrophoresis of poly ADP-ribosylated nuclear polypeptides extracted from cardiocyte nuclei that were isolated from normal and T_3 treated rats. Poly ADP-ribosylation was performed in vitro with isolated cardiocyte nuclei. The left part (A) shows results obtained from controls and the right (B) after T_3 treatment. Left ordinate is the radioactivity of poly(ADP-R); right ordinate the A_{570} of stained protein bands and abscissa indicates gel slices with

molecular weight markers (\bullet = radioactivity: — A_{570}). It is apparent from A that non histone proteins are predominantly poly ADP-ribosylated in vitro in controls, and histones only much less so. The largest diminution of poly ADP-ribosylation by in vivo T_3 treatment occurred with 130, and 65-90 kd molecular mass polypeptides. Present research is concerned with further discrimination of these proteins and with the effect of poly ADP-ribosylation on their specific DNA binding characteristics.

Fig. 7.



d. The role of poly(ADP-R) in human carcinogenesis.

A striking and sustained increase in poly ADP-ribosylation of non histone proteins was observed in the chemical carcinogen induced precancerous state (10) and we found recently that the promoter process of carcinogenesis (36) itself chronologically coincides with this increase of poly ADP-ribosylation even in the absence of a carcinogen. It was postulated that selective inhibition of poly ADP-ribosylation should prevent carcinogenesis by removing the DNA repair inhibiting (36) effect of increased poly ADP-ribosylation. This prediction was verified with a variety of carcinogens using human fibroblast transformation as a bioassay (15).

These undoubtedly biologically significant results demonstrate a readily recognizable function of poly(ADP-R) in cell biology that has an immediate significance in cancer chemotherapy, since it is predictable that carcinogenic action of chemotherapeutic agents will be abolished by the simultaneous

Administration of benzamide. This urgent problem is being pursued.

TABLE III. (cf. 37)

Prevention of carcinogenesis in human fibroblasts by 1mM benzamide, a specific inhibitor of poly(ADP-R) polymerase.

No.	Treatment of cells **	No. of cancer cell colonies formed / 50,000 cells
1.	Methylazoxy-methanol-acetate	300 - 350
2.	1 + benzamide	1
3.	N-me-N-nitrosoguanidine	45 - 50
4.	3 + benzamide	1
5.	3-hydroxy-1-propane sulfonic acid β-propiolactone	40 - 42
6.	5 + benzamide	1
7.	Benzamide	1

** Carcinogenesis was induced by exposing human fibroblast after release from G₁ block for 10 - 14 hours to 1 μM carcinogens. The preventive effect of benzamide runs parallel with its inhibitory effect of poly(ADP-R) synthetase, which enzyme system is greatly increased at this stage.

CONCLUSIONS. The biological function of poly(ADP-R) based on its macromolecular properties is envisaged as a nucleic acid component of a cross linking system, capable of promoting or inhibiting the regulatory effect of chromatin proteins on transcription. Two examples, the action of triiodothyronine and of chemical carcinogens illustrate this complex action of the homopolymer functioning as a protein modifier.

Although DNA and RNA can be profitably studied in isolated systems without paying attention to poly(ADP-R), integration with cellular physiology makes it mandatory to include poly(ADP-R) as a nucleic acid that possesses exclusively regulatory function.

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