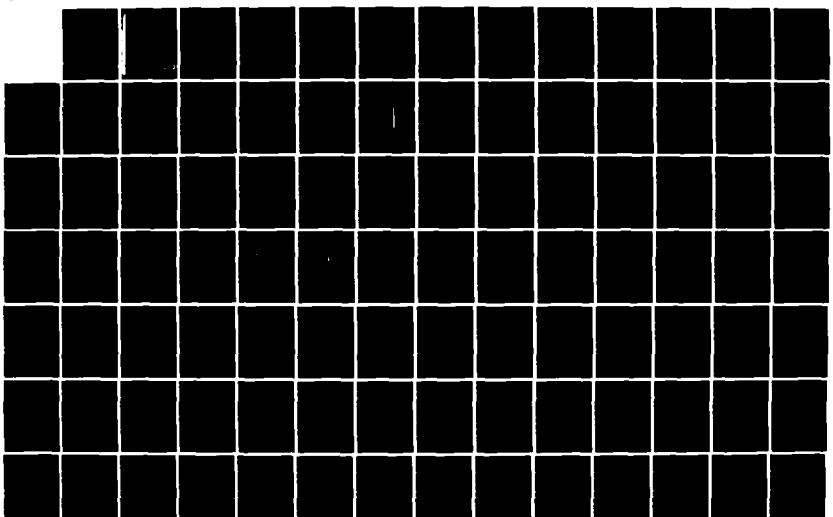
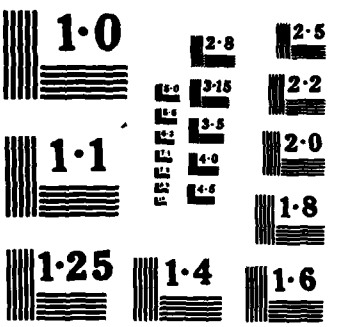
AD-A154 415 MOLECULAR TOXICOLOGY OF CHROMATIN: THE ROLE OF POLY(ADP-RIBOSE) IN GENE CONTROL(U) CALIFORNIA UNIV SAN FRANCISCO E KUN FEB 85 AFOSR-TR-85-0467 1/2

UNCLASSIFIED F49620-81-C-0007 F/G 6/20 NL




1·0
1·1
1·25
1·4
1·6
2·8
3·15
3·5
2·5
2·2
2·0
1·8

AFOSR-TR- 85-0467

(2)

Progress Report
(AFOSR - F - 49620-81-C-0007)

"Molecular toxicology of chromatin: the role of poly(ADP-ribose) in gene control"

Period: October 1983 through December 31, 1984, including no cost extension: September 31 - December 31, 1984).

Submitted by

Professor Ernest Kun

University of California, San Francisco

The Cardiovascular Research Institute
and Department of Pharmacology

(written in February 1985.)

85

UNCLASSIFIED

SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

1a. REPORT SECURITY CLASSIFICATION: UNCLASSIFIED	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION/AVAILABILITY OF REPORT: Approved for public release; distribution unlimited.
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S)	5. MONITORING ORGANIZATION REPORT NUMBER(S): AFOSR-TR- 85-0467
6a. NAME OF PERFORMING ORGANIZATION: University of California, SF; 6b. OFFICE SYMBOL (If applicable):	7a. NAME OF MONITORING ORGANIZATION: AFOSR/NL
6c. ADDRESS (City, State and ZIP Code): 103 Surge, 3rd and Parnassus, San Francisco, CA 94143	7b. ADDRESS (City, State and ZIP Code): Building 410, Bolling AFB, DC 20332-6448
8a. NAME OF FUNDING/SPONSORING ORGANIZATION: Air Force Office of Sci. Research; 8b. OFFICE SYMBOL (If applicable): NL	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER: F49620-81-C-0007
8c. ADDRESS (City, State and ZIP Code): Bolling Air Force Base, Washington, D.C. 20332-6448	10. SOURCE OF FUNDING NOS.

PROGRAM ELEMENT NO.	PROJECT NO.	TASK NO.	WORK UNIT NO.
61102F	2312	A5	

11. TITLE (Include Security Classification): Molecular toxicology of chromatin: the role of poly(ADP-ribose) in gene control

12. PERSONAL AUTHOR(S): Ernest Kun

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Yr., Mo., Day)	15. PAGE COUNT
Annual	FROM 10/83 TO 12/31/84	February 1985	136

16. SUPPLEMENTARY NOTATION: This report pertains to the following research projects:)

17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB. GR.	

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

I. Chemical and macromolecular structure of poly(ADP-ribose) 1. HPLC-isolation of poly(ADP-ribose.). 2. Fractionation, size analysis branching of poly (ADP-ribose) by HPLC and chemical analysis of subunits. 3. Mathematical model of polymerization of ADP-ribose. II. Biological and cell structural studies of poly(ADP-ribosylation in cellular and subcellular systems. III.Molecular studies on purified poly(ADP-ribose) polymerase system. 1. DNA-association of benzamide. 2. The role of lysine residues in the catalysis and DNA binding of poly(ADP-ribose) polymerase. IV. Cell transformation and poly ADP-ribosylation. 1. Inhibition of carcinogen initiated transformation. 2. Ultraviolet light induced transformation and its inhibition.

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION
UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT. ☒ DTIC USERS ☐	UNCLASSIFIED

22a. NAME OF RESPONSIBLE INDIVIDUAL	22b. TELEPHONE NUMBER (Include Area Code)	22c. OFFICE SYMBOL
Christopher Lind, Lt Col, USAF	(202) 767-5021	NL

DD FORM 1473, 83 APR EDITION OF 1 JAN 73 IS OBSOLETE.

UNCLASSIFIED

SECURITY CLASSIFICATION OF THIS PAGE

Progress Report
(AFOSR - F - 49620-81-C-0007)

"Molecular toxicology of chromatin: the role of poly(ADP-ribose) in gene control"

Period: October 1983 through December 31, 1984, including no cost extension: September 31 - December 31, 1984).

Submitted by

Professor Ernest Kun

University of California, San Francisco

The Cardiovascular Research Institute
and Department of Pharmacology

(written in February 1985.)

Published papers during the grant year

1. Kun, E., Kirsten, E., Milo, G.E., Kurian, P., Kumari, H.L. (1983) "Cell Cycle Dependent Intervention by Benzamide of Carcinogen Induced Neoplastic Transformation and in vitro poly ADP-ribosylation of Nuclear Proteins in Human Fibroblasts." Proc. Natl. Acad. Sci. USA, Vol. 80, pp 7219-7223.

2. Hakam, A., McLick, J. and Kun, E. (1984) "Separation of Poly(ADP-ribose) by High Performance Liquid Chromatography" J. Chromatography 296, 369-377

3. Griffin, M.J., Kirsten, E., Carubelli, R., Palakodety, R.B., McLick, J. and Kun. E. (1984) "The In Vivo Effect of Benzamide and Phenobarbital on Liver Enzymes: Poly(ADP-ribose)Polymerase, Cytochrome P-450, Styrene Oxide Hydrolase, Glutathione S-transferase and UDP-glucuronyl Transferase". Biochem. Biophys. Res. Comm. 122, 770-775.

4. Jackowski, G. and Kun, E. (1983) "The Effect of In Vivo Treatment with Triiodothyronine on the In Vitro Synthesis of Protein-poly(ADP-ribose) Adducts by Isolated Cardiocyte Nuclei." J. Biol. Chem. 258, 12587-12593.

5. Kun, E. et al. "Biochemical Basis of the Regulatory Role of Poly(ADP-ribose)" in Adv. Enzyme Regul. vol. 21 (Ed. Weber, G. Pergamon press, Oxford), (1983) 177-199.

6. Kun, E. "Macromolecular Properties of Poly(ADP-ribose) and the Regulation of its Biosynthesis and Degradation of Permeabilized Rat Brain Gliosarcoma 9L-Cells." Posttranslational Covalent Modification of Proteins (Ed. Johnson, B.C. Acad. Press, N.Y.), 1983, p. 397-416.

7. Milo, G.E., Kurian, P., Kirsten, E. and Kun, E. (1985) "Inhibition of Carcinogen Induced Cellular Transformation of Human Fibroblasts by Drugs that Interact with the Poly(ADP-ribose) Polymerase System. Initial evidence for the Development of Transformation Resistance." FEBS Letters 179, 332-336.

Accepted papers now in press

8. Minaga, T., and Kun, E. (1985) "Probable Helical Conformation of Poly(ADP-ribose) Proceedings of the VIIth International Symposium of ADP-ribosylation Reactions. Springer Publ. in press.

9. Hakam, A., and Kun, E. (1985) "Studies on the Structure of Poly(ADP-ribose) by HPLC. Separation of In Vitro Generated Polymer Chains, Analysis of Chain Length and Branching". Proceedings of the VIIth International Symposium on ADP-ribosylation Reactions. Springer Publ. (in press).

10. Bauer, P.I. and Kun, E. (1985) "The Role of Lysine Residues in the Catalytic Function and DNA Binding of Poly(ADP-ribose) Polymerase, Determined by the Covalent Modification of the Enzyme Protein with Methyl Acetimidate." Proceedings of the VIIth International Symposium on ADP-ribosylation Reactions. Springer Publ. (in press)

11. Kun, E., and Bauer, P.I. (1985) "Specific Binding of Benzamide to DNA; Possible Correlation to Antitransforming Activity". Proceedings of the VIIth International Symposium on ADP-ribosylation reactions. Springer Publ. (in press)

Abstracts

1. Sadeghi, M., Kirsten, E. and Kun, E. (1985) "Selective Augmentation of poly(ADP-ribose)Synthetase in SV_{40} Transformed 3T3 Cells, Exposed for 24 h. to Methotrexate + Benzamide." (FASEB, Anaheim, CA. May 1985).

2. Bauer, P.I., Hakam, A., and Kun, E. (1985) "4-diazo-benzamide: an Affinity Label for Poly(ADP-ribose) Polymerase" (FASEB, Anaheim, May 1985)

3. Tseng, A., Jr., Brooks, M., Cadman, E., Kirsten, E. and Kun, E.: Poly (ADP-ribose) Polymerase Activity and Inhibition of a Temperature Sensitive tsNY68 Rous Sarcoma Virus Infected Rad Kidney Cell Line by Benzamide (Amer. Assoc. Cancer Res., May 1985)

Invited Participation
International Conferences

1. "International Symposium on HPLC of proteins and nucleic acids" (Kun and Hakam), Nov. 1983, Monte Carlo
2. "Round table discussion: "Molecular Biology of Cardiac Cells", NIH Symposium, May 1984, Bethesda, MD (Kun)
3. American Heart Association Symposium: Biology of Cardiac Tissue" Snowmass, Colorado, August 1984 (Kun)
4. "VIIth International Symposium on ADP-ribosylation Reactions", Vitznau, Switzerland, October 1984. (Kun, Hakam, Minaga, Bauer, Sadeghi)

Invited Speaker on Departmental Seminars other than UCSF (E. Kun)

1. Austrian Research Centre, Seibersdorf, Austria, November 1983
2. Department Biochemistry, Cambridge University (UK), May 1984
3. Department Biochemistry, University of Sussex, November 1983
4. Department of Biochemistry, University Freiburg (Germany) May 1984
5. Institute for Enzyme Research, University of Wisconsin, August 1984

Invited Contribution of a Book Chapter in

Biochemical Actions of Hormones, Ed. Litwack, G., Acad. Press title, "Possible Participation of Nuclear Poly ADP-ribosylation eactions in hormonal mechanisms." by E. Kun et al. (due March 1, 1985)

Table of Contents of Research

I. Chemical and macromolecular structure of poly(ADP-ribose).

1. HPLC-isolation of poly(ADP-ribose).

2. Fractionation, size analysis branching of poly(ADP-ribose) by HPLC and chemical analysis of subunits.

3. Mathematical model of polymerization of ADP-ribose.

1. HPLC-isolation of poly(ADP-ribose).

SUMMARY

The homopolymer of ADP-ribose, poly(ADP-ribose), was synthesized *in vitro* by liver nuclei from NAD. The protein-poly(ADP-ribose) adducts were isolated and, after base hydrolysis or proteolysis by proteinase K, the free polymers were separated from NAD, ADP-ribose, AMP and adenosine, and quantitatively determined by reversed phase chromatography on an Ultrasphere ODS 5μ column. Oxidation of the polymer by $NaIO_4$ and labeling with ^3H by borotritiation maintained the polymeric structure, but its modification was detectable by the chromatographic system employed.

INTRODUCTION

The NAD-derived nuclear polymer of eukaryotic cells, poly(ADP-ribose) [1,2,3] has been recently identified to exhibit a specific helical conformation [4,5,6]. Covalent binding of this unique nucleic acid-like macromolecule to prevalently nuclear non-histone proteins [7,8,9] may represent a supramolecular chromatin network system that appears to influence programmed gene expression as detected by the regulation of cellular phenotype of human fibroblasts [10]. Further study of the physiological function of poly-(ADP-ribose) critically depends on specific techniques capable of determining the polymer in various cellular systems. Analytical methods which depend on the assay of the enzymatic degradation products, phosphoribosyl-AMP and AMP [11,12] cannot estimate the chain length of the polymers directly, only as a calculated average value, and it seems desirable to determine poly(ADP-ribose) without its degradation.

We have in the past employed immunochemical [7] and affinity chromatographic [8] methods for the assay of polymers in tissues. The disadvantage of these procedures is their relatively laborious nature.

In the present report we describe the development of a direct chromatographic technique for the determination of *in vitro* generated poly(ADP-ribose) on the basis of a direct separation of the polymers from nucleotides by high perfo- nce liquid chromatography (HPLC). *In vitro* tritiation of the intact polymers provides a sensitive technique for the direct assay of unlabeled poly(ADP-ribose).

EXPERIMENTAL

Materials.

^{14}C-NAD (269 mCi/mMol) was purchased from Amersham (Arlington Heights,IL, USA), [^{3}H] $NaBH_4$ (270 mCi/mMol) from New England Nuclear (Boston, MA, USA),

and NAD, Tris-HCL, Proteinase K, and $NaIO_4$ from Sigma Chemical Co. (St. Louis, MO, USA). Boronic acid gel (Affi-Gel 601) was obtained from Bio-Rad (Richmond, CA, USA). Ammonium carbonate and potassium phosphate, both HPLC grade from Baker Co. and Fisher Co., respectively. All other chemicals used were reagent grade. Analytical methods were the same as detailed previously [4,5].

Large scale preparation of poly(ADP-ribose) for HPLC standards.

The method of the enzymatic synthesis was the same as described [4,5,6], except the incubation system was scaled up to 300 mL, NAD concentration (spec. radioactivity 74.9 cpm/mMol) was increased to 5mM, and the temperature of incubation was raised to 37° for 60 min. The incubation buffer presently used was 100 mM Tris-HCL (adjusted to pH 8.5 at 25°C), 67 mM sucrose, 10 mM $CaCl_2$, 0.8 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.2 mM EDTA, 0.1 mM phenylmethyl-sulfonyl flouride and rat liver nuclei, prepared by a published method [13] except Triton X-100 was omitted. The concentration of nuclear protein in the incubate was 3.2 mg/ml. Further modifications were: hydrolysis of protein poly(ADP-ribose) bonds in 14 mL 1 N NaOH for 3 hours (instead of overnight), the use of Diaflo ultrafilters with 1 kDa cutoff, and the quantitative elution of poly(ADP-ribose) from the boronate resin (15 g, 3 cm x 6 cm column) with 0.5 M Tris HCL (pH 7.0)* a method superior to previous procedures [4,5]. In this system, 14.7% NAD was converted to poly(ADP-ribose), with a recovery of spec. radioactivity within $\pm$7% of the originally added labeled NAD.

---

*This modification was suggested by Dr. Takeyoshi Minaga.

Enzymatic synthesis of poly(ADP-ribose) in small incubates, used for experimental HPLC separations.

For individual HPLC experiments the preparative incubate was proportionally reduced to 0.1 ml, the NAD concentration to 2.5 mM and the specific radioactivity increased to 1700 cpm/n mol. Variations in incubations are given in results. Reactions were stopped either by (A) 10% $HClO_4$ (final conc.) at 4°, perchlorate removed by $KHCO_3$ or (B) by proteinase K added in two portions (100 µg/mg nuclear protein) and incubation for 2 x 20 min. at 37°. In (A) adducts were hydrolyzed by 1 M NaOH by incubation at 55° for two hours and pH adjusted to 6.0 subsequently.

Determination of chain length was done by molecular filtration [7] and calibrated by quantitation of phosphoribosyl-AMP/AMP (after enzymatic digestion) in each elution peak by quantitative paper chromatography [14].

Oxidation of poly(ADP-ribose) with $NaIO_4$ and reduction with [^{3}H]$NaBH_4$.

This procedure is an adaptation of a published method [15]. To 0.1 mL of a 3.6 mM solution of poly(ADP-ribose) in distilled water was added 0.08 mL of 10 mM $NaIO_4$, and the mixture incubated in the dark at room temperature for 1 hour. Then 0.04 mL of potassium phosphate buffer (0.10 M, pH 6.8) followed by 0.02 mL of a 0.10 M solution of [^{3}H]$NaBH_4$ in 0.10 M KOH were added in sequence, and the mixture incubated in the dark at room temperature for an additional hour. In order to decompose excess borotritiide, 0.10 mL of aqueous acetic acid (2.0 M) was added and the mixture allowed to stand under a fume hood for 1 hour. The solution was then evaporated to dryness under a stream of nitrogen at room temperature, and the residue dissolved in 0.2 mL of distilled water to give an aqueous solution suitable for UV spectroscopy and HPLC analysis. Using [^{3}H]$NaBH_4$ of a specific

radioactivity of 270 mCi/mMol, the specific radioactivity of the resultant tritiated poly(ADP-ribose) was in range 63-93 mCi/mMol, corresponding to an average of 58% of non-exchangeable ^{3}H per mol of $NaIO_4$-reactive residues per ADP-ribose units.

HPLC

Chromatography was performed with the following components: Waters Associates (Milford, MA, USA) Model 6000A solvent delivery pumps, a Waters Model 680 Gradient Controller, a Waters Model 730 Data Module, a Beckman-Altex (Berkeley, CA, USA) Model 155 Variable Wavelength Detector, a Radioactive Instrument and Chemical Co. (Tampa, FL, USA) Flo-One Model HP radioactivity Flow Detector, and a Beckman-Altex analytical reversed phase column (Ultrasphere ODS, 5 μ, 4.6 mm x 25 cm), with a pre-column packed with the same sorbent as the analytical column. Chromatography was carried out at ambient temperature. Prior to use the column required pretreatment by washing with buffer C of System I (about 200 mL) at a rate of 0.5 mL/min. Two solvent gradient programs were utilized to elute poly(ADP-ribose) and/or nucleotides. System I uses three buffers: A, 0.1 M potassium phosphate (pH 4.25); B, the same as A but containing 20% methanol; C, 0.1 M potassium phosphate (pH 7.0), 1 M urea, and 50% acetonitrile. Flow rate was 2.0 mL/min throughout. Upon sample injection the gradient is started from 100% A to 100% B in 20 minutes using concave gradient curve 9 (Figure 2). Elution continues at 100% B for an additional 2 min. and then a linear gradient is commenced from 100% B to 100% C during 6 min., and elution continued at 100% C for about 10 minutes. System II uses two buffers: A, 0.1 M potassium phosphate (pH 7.0), 1 M urea; B, same as A but containing

50% acetonitrile, and the flow rate was 3.0 mL/min. Upon sample injection gradient is started from 100% to 80% A and 0% B to 20% B in 60 minutes, using a linear gradient (Δ1% per 3 min.).

RESULTS AND DISCUSSION

Under the preparative conditions of the polymer synthesis the preponderance of medium chains was apparent, corresponding to an average mol. weight of 5000 to 20,000, an average length of 8-30 ADP-ribose units {7,14}.

The HPLC elution pattern of *in vitro* tritiated medium size polymers by System I is shown in Figure 1. Apart from a ^{3}H-containing impurity (elution at 2-3 min.) the polymer emerges as a symmetrical peak between 27 and 30 minutes. Figure 1 also demonstrates that no contamination with nucleotides was present, since during the first 25 minutes of elution by System I no nucleotide was detected. The ^{3}H-labeled polymer was collected as HPLC eluate and its apparent molecular size compared to that of the parent poly(ADP-ribose) fractions which were labeled only with ^{14}C in the adenine moiety and had not been subjected to borotritiation. As shown in Figure 2, there is a good agreement between the macromolecular profile of the tritiated fraction isolated by HPLC and the parent sample, therefore the oxidation and reduction procedure had no influence on the macromolecular nature of the polymer. Borotritiation had no effect on the absorption spectrum of poly(ADP-ribose) either. However, some degree of structural modification due to the opening of the constituent ribose rings could be detected by reversedphase HPLC of short (3-10 ADP-ribose units) and medium (8-30 ADP-ribose units) oligomers {Fig. 3 (a) and (b)} in terms of changed retention times.

The specificity of the HPLC method was tested by determining the inhibitory effect of 10mM benzamide (cf10) on the *in vitro* synthesis of poly(ADP-ribose). Poly(ADP-ribose) was generated by small-scale enzymatic incubates (see methods)

and chromatographed using System I. The results of these experiments are shown in Figure 4. The difference between (a) and (b) consists of the inhibition of the polymerase by benzamide exemplifying the role of the enzyme in (b), corresponding to an almost complete absence of poly(ADP-ribose). A comparison of methods capable of quantitative release to the free polymer from covalent binding to proteins is illustrated in Figure 5. In Figure 5a polymers were released from covalent bonds to proteins by base hydrolysis and in Figure 5b by treatment with proteinase K, and analyzed by System I. Based on the areas determined under poly(ADP-ribose) in Figure 5a and 5b, the difference between the two methods was found to be negligible(see Table), thus from the viewpoint of the analytical method they are equivalent. It is of interest that the phosphoribosyl-AMP could not be detected in nuclear incubates, thus no significant pyrophosphorolytic degradation of the polymer is apparent. The AMP and adenosine are formed from ADP-ribose as determined in separate experiments (not shown). The elution position of authentic phosphoribosyl AMP is indicated in Figure 1.

As shown in the Table, there is a small amount of $HClO_4$-soluble poly (ADP-ribose) as determined by HPLC (No. 1.). This fraction may contain oligopeptides covalently attached to the polymer which could be the result of trace proteolysis of polymer-protein adducts. This question is under investigation. The presence of benzamide (No. 2) results in profound inhibition of polymer synthesis, determined either by HPLC or by acid precipitation. The ADP-ribose increments found under these conditions (No. 2) and after varying incubation (Nos. 3,4) indicate NAD-glycohydrolase activities that are probably unrelated to the polymerase reaction. When polymer-protein adducts are hydrolyzed by NaOH (No. 5) both NAD and ADP-ribose are degraded to AMP, as would be expected, but the yield of poly(ADP-ribose) is the same after hydrolysis of polymer-protein adducts by proteinase K (compare 4

and 5), determined either by HPLC or by isotope analysis of acid precipitable products before hydrolysis. This method of HPLC analysis of poly(ADP-ribose) is being presently applied for the assay of endogenous polymers in animal tissues under varying experimental conditions.

REFERENCES

1. Chambon,P., Weill,J.D., Dolly,J., Strosser,M.T. and Mandel,P. Biochem. Biophys. Res. Commun. 25 (1966) 638.

2. Fujimura,S., Hasegawa,S. and Sugimura ,T. Biochem. Biophys. Acta 134 (1967) 496.

3. Nishizuka,Y., Ueda,K., Nakazawa,K. and Hayaishi,O. J. Biol. Chem. 242 (1967) 3164.

4. Minaga,T., McLick, J., Pattabiraman,N. and Kun,E. J. Biol. Chem. 257 (1982) 11942.

5. Minaga,T. and Kun,E. J. Biol. Chem. 258 (1983) 725.

6. Minaga,T. and Kun,E. J. Biol. Chem. 258 (1983) 5726.

7. Minaga,T., Romaschin,A.D., Kirsten,E. and Kun,E. J. Biol. Chem. 254 (1979) 9663.

8. Romaschin,A.D., Kirsten,E., Jackowski,G. and Kun,E. J. Biol. Chem. 256 (1981) 7800.

9. Jackowski,G. and Kun,E. J. Biol Chem. 258 (1983) 12587.

10. Kun,E., Kirsten,E., Milo,G.E., Kurian,P. and Kumari,H.L. Proc. Natl. Acad. Sci. USA 80 (1983) 7219.

11. Sims,J.L., Juarez-Salinas,H. and Jacobson,M.K. Anal. Biochem. 106 (1980) 296.

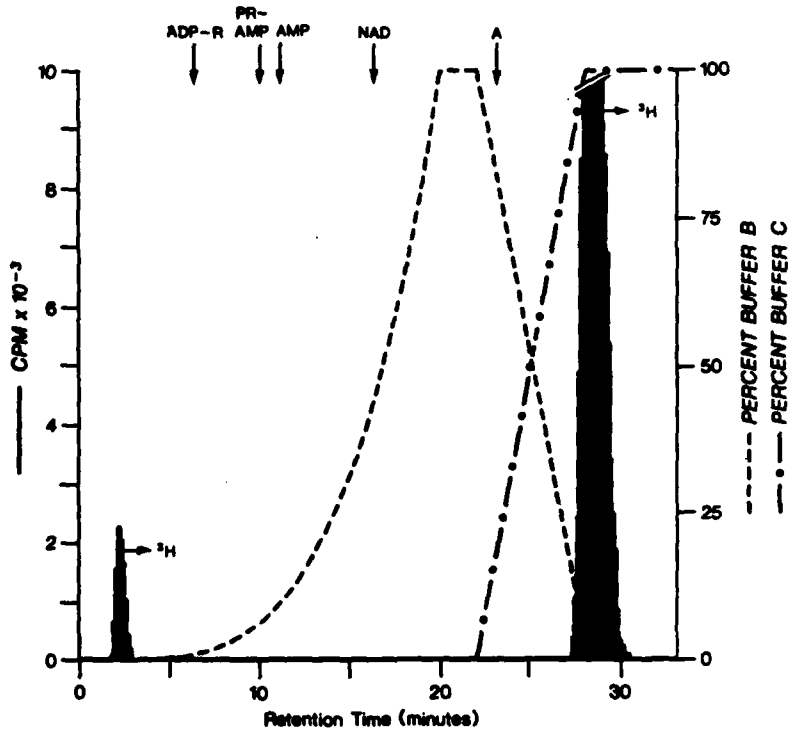
12. Juarez-Salinas,H., Mendoza-Alvarez,H., Levi,V., Jacobson,M.K. and Jacobson,E.L. Anal. Biochem. 131 (1983) 410.

13. Liew,C.C. and Chan,P.K. Proc. Natl. Acad. Sci. USA 76 (1976) 3458.

14. Shima,T., Fujimura,S., Hasegawa,S., Shimizu,Y. and Sugimura,T. J. Biol. Chem. 245 (1970) 1327.

15. Randerath, E., Yu, C.T., , and Randerath, K. Anal. Biochem 48 (1972) 172.

Fig 1.

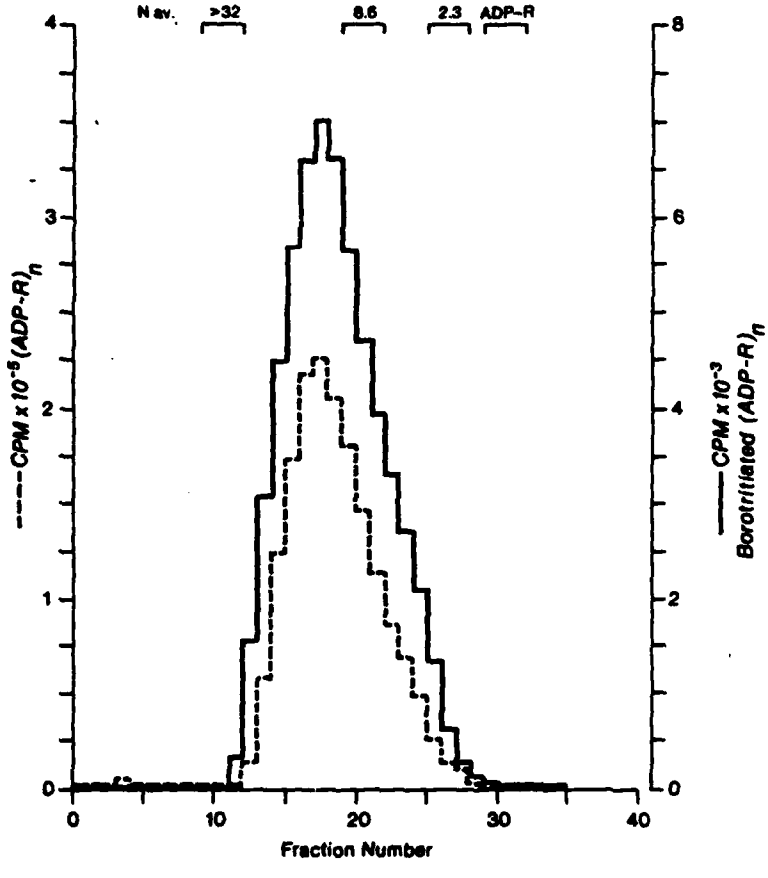


top: ADP-R = adenosine diphosphoribose

PR-AMP = phosphoribosyl-AMP

A = adenosine.

Fig 2



(Top). $N_{av.}$ = number average of ADP-ribose units.

Fig 3,

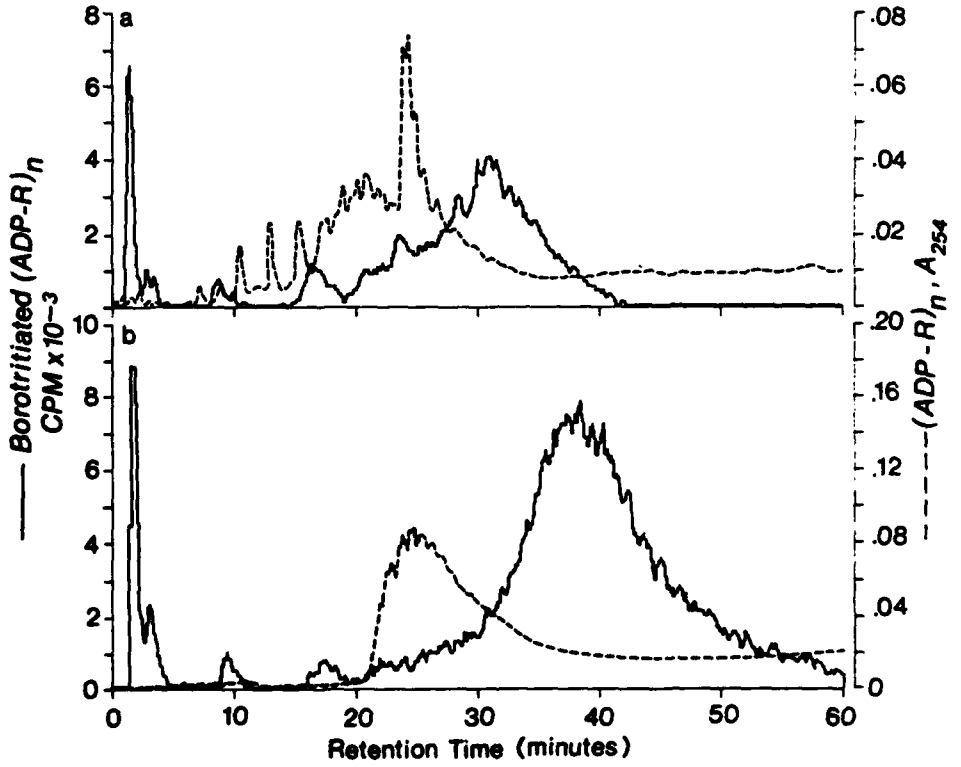

a
b
8
6
4
2
10
0
.08
.06
.04
.02
.20
.16
.12
.08
.04
0
0
10
20
30
40
50
60
Retention Time (minutes)
— Borotritiated (ADP-R)n CPM x 10−3
----(ADP-R)n, A254

Fig 4.

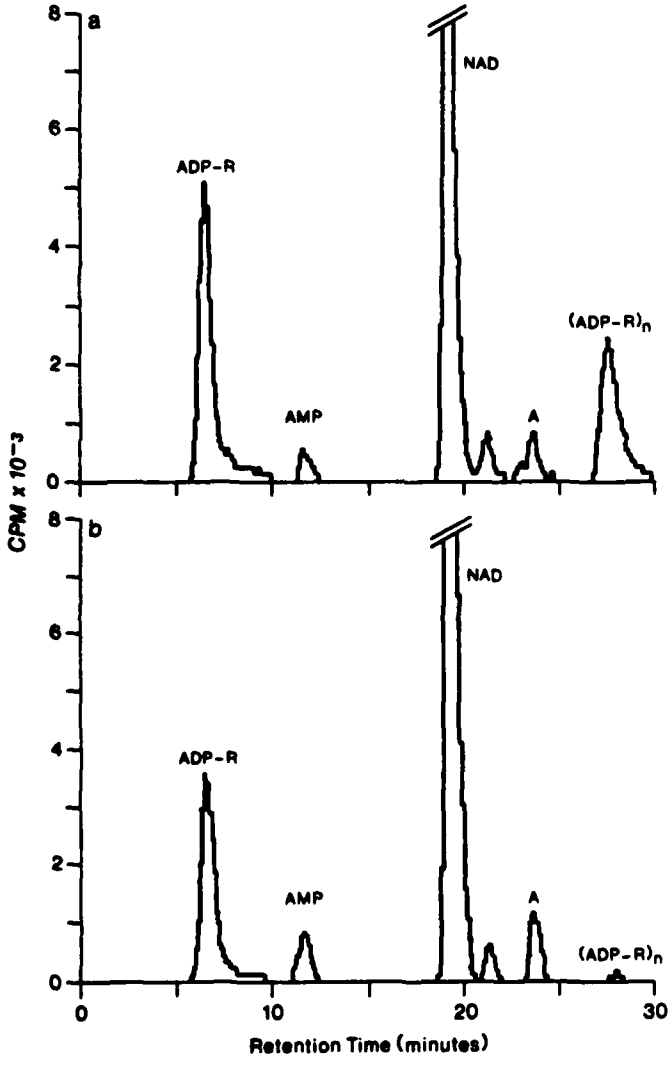
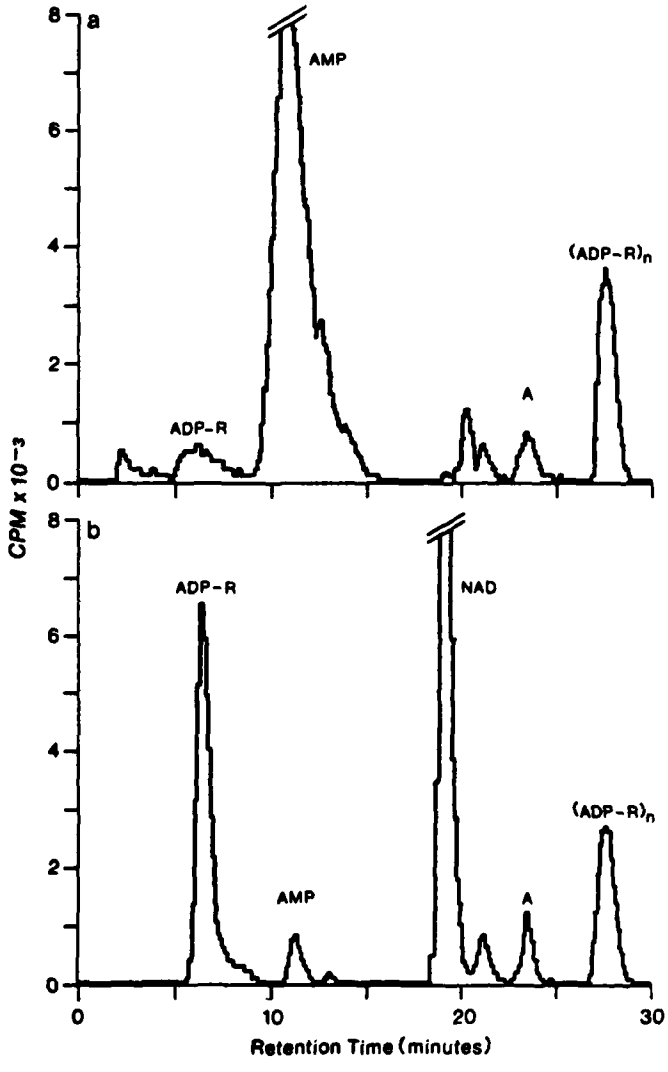

a
b
NAD
ADP-R
AMP
A
(ADP-R)n
CPM x 10⁻³
0
2
4
6
8
0
10
20
30
Retention Time (minutes)

Fig 5.


a
AMP
ADP-R
A
(ADP-R)n
b
ADP-R
NAD
AMP
A
(ADP-R)n
CPM x 10⁻³
0
2
4
6
8
0
10
20
30
Retention Time (minutes)

TABLE

Quantitative distribution of NAD, its enzymatic products and poly(ADP-ribose) determined under varying conditions. Results are expressed as % conversion of NAD to products.

No.	Experimental Conditions	Nucleotides				Poly(ADP-ribose)	
		ADP-R	5'-AMP	NAD	Adenosine	Determined by HPLC	Determined by radioactivty of acid ppt.
1.	$HClO_4$-soluble	11.6	1.5	80.7	0.9	2.9	----
2.	20 mintue incubation* with benzamide	15.4	3.3	75.3	3.1	0.8	0.1
3.	20 minute incubation*	23.1	2.5	53.2	2.6	14.7	12.7
4.	40 minute incubation*	26.4	3.1	48.2	4.0	12.7	13.5
5.	40 minute incubation followed by NaOH treatment	1.4	71.0	0.4	2.9	12.4	13.5

*hydrolized by proteinase K

LEGENDS TO FIGURES

Figure 1. Reversed phase HPLC purification of $NaIO_4/[^3H]NaBH_4$-treated poly(ADP-ribose). The main peak (95% of the total radioactivity) was collected and characterized (see Figure 3). HPLC elution System I was employed. ------- = % buffer B; - - - - = % buffer C; shaded area = eluted material.

Figure 2. Elution profile on Sephadex G-50 of medium chain length poly(ADP-ribose) before and after $NaIO_4/[^3H]NaBH_4$ treatment. ---- = before; ——— = after.

Figure 3. Reversed phase HPLC analysis of (a) short chain length and (b) medium chain length poly(ADP-ribose). The same samples were analyzed before (UV detection. ----- = right ordinate) and after borotritiation (cpm ——— = left ordinate), indicating that treatment with $NaIO_4$ + $[^3H]NaBH_4$ increases retention time. In (a) 4.6×10^5 cpm and in (b) 1.1×10^6 cpm were injected, and elution System II was employed.

Figure 4. Reversed phase HPLC analysis of ^{14}C-labeled products from incubation of rat liver nuclei with ^{14}C-NAD in the absence (a) and the presence (b) of 10 mM benzamide. The incubations were for 20 min., followed by treatment with proteinase K. In (a) 1.74×10^5 cpm and in (b) 1.82×10^5 cpm were injected and elution System I was used. A = adenosine. There is a trace of unknown component following NAD.

Figure 5. Reversed phase HPLC analysis of ^{14}C-labeled products from incubation (40 min.) of rat liver nuclei with ^{14}C-NAD followed by treatment with (a) 1 M NaOH and (b) proteinase K. In (a) 2.59×10^5 cpm and (b) 2.07×10^5 cpm were injected and elution System I was used.

2. Fractionation, size analysis, branching of poly(ADP-ribose) and chemical analysis of subunits.

Summary

Separated macromolecular fractions of *in vitro* synthesized poly(ADP-ribose) by liver nuclei were subjected to ion-exchange chromatography in a programmed HPLC elution system. The effects of ionic strength, pH and temperature on the separation of poly(ADP-ribose) chains were determined. Short chain oligomers (up to n = 11) were fractionated into individual components by base line separation. Each fraction was analyzed for chain length. Trace amounts of Ado(P)Rib(P)Rib(P) found in phosphodiesterase digests were taken as indication of apparent branching. In phosphodiesterase digests of the shorter oligomers, besides traces of the above component, two other digestion products were also observed, presumably representing oligomer termini, one terminal fragment being dominant in short oligomers. Medium and long chain oligomers were partly resolved to individual components, and especially the long oligomers exhibited marked temperature dependent elution patterns. Apparent branching increased with increasing chain length up to about 3% for n = 44 and components presumably indicating termini diminished to mere traces. The adenine spectra of all fractions identified individual components.

Introduction

Structural aspects of poly(adenosine diphosphate ribose), poly(ADP-ribose), the nucleic acid like homopolymer derived from NAD in cell nuclei, that is covalently bound to chromatin proteins, have been reviewed(1,2). The most conspicuous property of the isolated homopolymer is its secondary structure (3,4) which predicts helix-helix interaction between single strands. A further structural complexity has been identified by the isolation of 2'-[2"-(5"'-phosphoribosyl)-5"-phosphoribosyl]-adenosine 5' monophosphate), Ado(P)Rib(P)Rib(P), as a variable, minor component of phosphodiesterase digest of poly(ADP-ribose), indicating branched structure (5,6). The Ado(P)Rib(P)Rib(P) product has been identified in 3T3 cells and various animal tissues (7,8) and an ubiquitous occurrence of variable branching proposed. Estimation of poly(ADP-ribose) in these studies has been based on the etheno derivatives of phosphodiesterase and alkaline phosphatase digestion products (9). Some degree of uncertainty regarding the reported analytical values is based on the apparent 10-20 fold variance between Ado(P)Rib(P)Rib(P) content of liver (on a DNA basis) as published by two different groups (compare ref. 6 with ref. 7). This discrepancy may not necessarily be related to differences in techniques only, and could reflect real biological variations which cannot be estimated at present. We have recently developed a high performance liquid chromatographic method, capable of direct separation of undegraded poly(ADP-ribose), a procedure based on reversed phase chromatography (10). The present worked is concerned with the resolution of isolated oligomeric fractions of $(ADP\text{-ribose})_n$

into components containing discrete chains. The main purpose of the present experiments was to compare the behavior of oligomers of varying chain length at varying temperatures during elutions from an ion exchange column while monitoring spectra. Enzymatic digestion products of the highly purified polymers, which were obtained by base hydrolysis of protein adducts, have been further analyzed and from the Ado(P)Rib(P)Rib(P) content a correlation between chain length and apparent branching was estimated. As yet structurally unidentified phosphodiesterase degradation products, presumably representing oligomer-protein end-pieces, were quantitatively isolated from short oligomers.

Experimental

Materials:

NAD, Tris, Proteinase K, alkaline phosphatase (from Bovine intestine Type VII-S) and ammonium sulfate were purchased from Sigma Chemical Co., (St. Louis, MO, USA). Crotalus adamanteus venom phosphodiesterase was obtained from Pharmacia P-L Biochemicals, Inc. (Piscataway, NJ, USA), boronic acid gel (Affi-Gel 601), acrylamide and bisacrylamide from Bio-Rad (Richmond, CA, USA). NAD[^{14}C]from Amersham (Arlington Heights, IL, USA). Ammonium carbonate and potassium phosphate, both HPLC grade, were purchased from Baker Co. and Fisher Co., respectively. All other chemicals were reagent grade.

HPLC:

HPLC was performed with the following components: Waters Associates (Milford, MA, USA) Model 6001 Solvent Delivery Pumps, Waters Model 680 Gradient Controller, Waters Model 730 Data Module, and Hewlett Packard (Santa Clara, CA, USA) Model 1040A High Speed Spectrophotometric Detector. Chromatographic data were stored in a Hewlett Packard Model 9121D Disc

Memory System and plotted by a Hewlett Packard HP 7470A Graphic Plotter. The columns employed were Synchrom, Inc. (Linden, IN USA) AX100 and AX300, depending on the nature of the analyses. Column temperature was maintained by a Waters temperature control accessory.

Solvent gradients involved at least two of the following buffer systems: buffer A, 0.05 M potassium phosphate (ph 4.25); buffer B, 0.05 M potassium phosphate (pH 4.25), 1 M ammonium sulfate, and 30% methanol (v/v); buffer C, same as B but containing o.2% (v/v) trifluoroacetic acid, (pH=2). Flow rate was 0.6 ml/min in all cases. Chain length analyses of poly(ADP-ribose) was based on spectral analyses of emerging fractions from the AX300 column with the following solvent gradient: 100% A to 100% B in 90 min (linear) and from 100% B to 100% C (linear) in 45 min. For average chain length determination degradation products of the oligomers by phosphodiesterase were analyzed on the AX100 column with the linear gradient from 100% A to 50% B, 50% A in 45 min.

Gel electrophoresis was performed by the method reported in ref. 11.

Alkaline phosphatase dephosphorylations were performed by the method reported in ref. 12.

Phosphodiesterase digestion of poly(ADP-ribose):

In 100 µl of incubation mixture containing 50 mM Tris-HCl (pH 7.8) and 5 mM $MgCl_2$, 2 A_{260} units of poly(ADP-ribose) were incubated with one unit of enzyme at 37°C for one hour. The mixture then was treated with 20µg of proteinase K at 37°C for an additional hour. Samples were directly injected into the HPLC without further deproteinization.

The chain length of polymers (n) was calculated by a modification of a published formula (13) as follows:

$$n = \frac{[AMP] + [PR\text{-}AMP] + [Ado(P)Rib(P)Rib(P)] + [End\ fraction^*]}{[AMP] - [Ado(P)Rib(P)Rib(P)]}$$

*PR-AMP denoted 2'-(5"-phosphoribosyl) adenosine 5'-monophosphate, and end fraction = probable attachment site to protein acceptors of as yet unknown structure (see Results and Discussion and Fig. 5.)

Preparation of poly(ADP-ribose):

The incubation system (300 ml total volume) for large scale polymer synthesis was composed of 100 mM Tris-HCl (pH 8.5 at 25°C), 67 mM sucrose, 10 mM $CaCl_2$, 0.8 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.2 mM EDTA, 0.1 mM phenylmethyl sulfonyl fluoride, and 5 mM NAD containing ^{14}C-labeled NAD with a specific radioactivity of 74.9 cpm/n mol. The quantity of rat liver nuclei isolated from male Wistar rats was 3.2 mg per ml of incubation mixture. Nuclei were isolated as reported (10). Following incubation for 60 min at 37°C in a shaking flask the reaction was terminated by addition of 32 ml of cold (4°C) 50% (w/v) trichloroacetic acid, centrifuged, and the pellet washed sequentially with two 170 ml, one 75 ml and one 37.5 ml portion of cold (4°C) 5% trichloroacetic acid. The radioactivity in the final wash (supernatant) was less than 8.4% of that in the sediment. The sediment was washed 3 times with 37.5 ml portions of diethyl ether, and, after removal of traces of ether by vacuum, it was dissolved in 14 ml of 1N NaOH, stirred for 3 hours, centrifuged, and the supernatant adjusted to pH 9 with 3M $(NH_4)_2CO_3$. This treatment yields free poly(ADP-ribose) which, after subsequent purification, contains no detectable traces of protein (4). Proteins were removed by extraction with 800 ml of H_2O-saturated phenol followed by centrifugation. The phenol phase was washed with 30 ml of aqueous 1 M $(NH_4)_2CO_3$, and the aqueous phases were combined, which contained 92% of the total radioactive material. Traces of phenol in the aqueous phase were removed by extraction with 100 ml of diethyl ether, and residual ether was removed by a stream of nitrogen. The resultant aqueous solution was passed through a 3 cm x 6 cm column (15g) of boronate resin (10) previously washed and equilibrated with 1 M $(NH_4)_2CO_3$. At a flow rate of 0.4 ml/min, fractions of 8 ml each were collected and monitored at 260 nm. When absorbance in the effluent reached baseline, elution with 0.5 M Tris-HCl (pH 7.0) was initiated. In contrast to elution with

H_2O (3), Tris-HCl recovered about 90% of the applied radioactivity from the boronate column . Fractions containing radioactivity were combined and passed through a Millipore membrane filter(0.1 μm pore size) to remove particles of the boronate resin, then concentrated on a Diaflo Ultrafilter (UM-2) having a molecular cut off of 1,000 daltons. About 7% of the radioactivity passed

Fig. 1 through the filter representing nucleotides. The supernatant was freeze-dried and applied to a Sephadex G-50 column. Figure 1 shows the macromolecular distribution of poly(ADP-ribose). Fractions 10-13 contain long chains, fractions 14-22 medium chains, and 23-36 short chains of poly (ADP-ribose). These pooled fractions were desalted by a Diaflo Ultrafilter (UM-2), where salt removal was monitored by conductivity measurements.The purity of the polymer at this stage has been established (3,4). The amounts of recovered polymers were: 20.3 mg of long chain, 48.2 mg of medium chain, and 13.0 mg of short chain poly(ADP-ribose), as determined by spectrophotometry (A_{260nm}), using $\varepsilon = 15 \times 10^3$ per monomeric unit. The specific radioactivity of the poly(ADP-ribose) was within 7% of that of the starting NAD, indicating no appreciable dilution by trace amounts of non-labeled endogenous NAD or polymer, or by trace amounts of other UV absorbing macromolecules. In this system 14.7% of NAD was converted to poly (ADP-ribose) as determined by acid precipitable radioactive material.

For highly radioactive poly(ADP-ribose) markers (26000 cpm per n mol) the concentration of NAD was reduced to 2.5 mM, without alteration of other components, as described above.

Results and Discussion

When unfractionated poly(ADP-ribose), sythesized to contain high radioactiv-

Fig. 2 ity as described in the Experimental section, was subjected to HPLC on an anion exchange column with a pore size of 300 Å using a ternary solvent gradient, elution patterns as shown in Fig. 2 were observed. During the first 90 minutes of the chromatogram, in which elution is effected by increasing ionic strength

(linear gradient from zero to 1.0 M $(NH_4)_2SO_4$), the early peaks show baseline separation from each other, but with longer retention times resolution decreases and is eventually lost at about 80 minutes. Then at 90 minutes, with ionic strength held constant, a linear pH gradient(0 to 0.2% trifluoroacetic acid in Buffer B) with a duration of 35 minutes is carried out. With elution being effected by decreasing pH, a single sharp peak at 118 minutes is observed. This peak is most probably a family of long chain polymers which are protonated, thus made uncharged at lower pH (see Buffer B), and are thus desorbed from the column at higher acidity (Buffer C). Following that peak no further radioactive material was eluted.

The exact reason for the loss of resolution with increasing chain lengths is not known but it can be assumed that the ion exchange separation is incomplete because with longer chain lengths the percentage charge difference between consecutive polymers diminishes. It is also apparent that with longer chain lengths there is a proportionately greater amount of branching in the polymer (see below), which adds to the complexity of the polyanion.

A correlation between pore size of the packing matrix and the resolution of oligomers was sought. It was found that pore size of 300 Å was optimal because at 100 Å smaller oligomers separated but resolution was lost above n = 10, and on the other hand between 300 Å and 1000 Å no improvements of resolution were detectable. Therefore, as a compromise, the pore size of 300 Å was chosen for the separation of all oligomers of ADP-ribose, whereas nucleotide digestion products (by phosphodiesterase) were chromatographed on column of a pore size of 100 Å.

Further studies were undertaken on poly(ADP-ribose) that had been fractionated on a Shephadex G50 column (see Fig. 1) into short, medium and long chains. Since the effects of temperature on the resolution and recovery of nucleic acids on ion exchange columns are well known, the effects of varying elution temperatures on the resolution of poly(ADP-ribose) fractions were determined.

Fig. 3

Fig. 3A shows the effect of increasing temperature on the resolution of the medium chain length polymer. By increasing from 29°C (a) to 50°C (b) and to 75°C (c), better resolution is achieved, the peaks become progressively sharper yet there is no apparent change in retention times. The same effects were observed with short chain poly(ADP-ribose) (not shown). There was no evidence of degradation of poly(ADP-ribose) at the elevated temperatures.

The effect of temperature on the behavior of long chain poly(ADP-ribose), however, is different (Fig. 3B). At 29°C (a), the long chain polymer, eluting in the pattern of a broad peak (Long I) and a sharp peak (Long II), is obtained with relatively low recovery. On going to 50°C (b) the sharp peak shows considerable increase in intensity. Further increase in temperature to 75°C (c) partly "melts" the sharp peak into the broad peak indicating a temperature dependence of both peaks. This type of behavior is partly predictable from the structural melting curves seen with isolated polymers (3,4).

The low recovery observed at 29°C was investigated further as follows. The sharp peak (Long II) in Fig. 3B was isolated and re-injected at 29°C yielding poor recovery as expected. Then, after the column had been washed and re-equilibrated with starting buffer at 29°C, the column temperature was elevated to 50°C and the elution gradient was repeated (without any further sample injection), resulting in the elution of long peak II that had been tightly absorbed at 29°C. Subsequent repetition of the gradient at higher temperature (75°C) did not result in the appearance of any further UV-absorbing material. It is evident that the composite of long chain polymers can be quantitatively recovered at an appropriate temperature (50°C to 75°C) but resolution beyond two composite peaks is not possible in this system, with the exception of Long peaks I and II which can be obtained in individual fractions by the manipulation described above. It should be noted that, in contrast, recoveries for short and medium chain lengths are essentially quantitative at

low temperature (29°C) as well as at higher temperatures. (50°C- 75°C).

The chain lengths of individual peaks were determined according to established methods (see Experimental procedures and ref. 13). A composite of individ-

Fig. 4 ual chromatograms obtained for short, medium and long polymers is shown in Fig. 4. HPLC resolution of short chains ($N_{average}$ = 6.96), that were eluted from Sephadex between fractions 23 to 36 (Fig. 1), is demonstrated in Fig. 4A, where each emerging peak was analyzed by its UV spectrum (A_{220} to A_{340}). Twelve major peaks separated (ending at retention time 63 min) and each fraction exhibited characteristic adenine absorbance shown in the inset spectra. For the short chain length group (top row of insets) two superimposed spectra were recorded in each plot and retention times are indicated in the upper right corner of the plot. Each major peak was collected and analyzed further. Peak I is AMP and peaks 2 to 4 are short oligomers (n 2-4). Peaks 5-10 were sufficient

Table for chain length analyses by phosphodiesterase digestion and the results of these analyses are given in the Table. Increasing chain length coincides with an increase in apparent branching from 0.34% (n = 7.1) to 0.5% (n = 11.0). Separation and spectral identification of peak fractions were carried out for medium ($n_{average}$ = 18.6, eluate fraction 14-22, see Fig. 1) and long ($n_{average}$ = 44.0, eluate fraction 10-13, see Fig. 1) size oligomers as illustrated in Figs. 4B and 4C, respectively. UV absorbance spectra for medium chain length peaks are shown in the middle row of insets (with three superimposed spectra in each plot) and those for long chain length peaks shown in the lower row of insets (one spectrum per plot). In all cases the retention times are indicated in the upper right corners of the plots.

Although partial resolution into individual peaks of medium chain length poly (ADP-ribose), $n_{average}$ = 18.6, is apparent and spectral identification of

each peak as adenine nucleotide clearly defines the oligomers, base line separation as obtained for short chains does not take place (Fig. 4B). However, more than 18 individual peaks are discernable but their individual chain lengths cannot be determined because of incomplete separation (see footnotes in Table).

As given in the Table, the average chain length of polymers eluting in long peaks I and II differ somewhat and apparent branching is increased from 2.37% to 3.1%. The most probable explanation for the behavior of long chains in this HPLC system is a simultaneous contribution of both inter-helical forces (3,4) and increased branching, producing a network of polymers that fails to be resolved on the basis of charge separation.

The evidence for branching was examined by separation of the products of phosphodiesterase digestion of poly(ADP-ribose) (Fig. 5). The lower part (5A) and upper part (5B) of the figure illustrate the resolution of digests of short and long chain polymers respectively. In both cases AMP and PR-AMP were identified, which are the result of cleavage of the terminal and internal pyrophosphate linkages respectively. The nucleotide Ado(P)Rib(P)Rib(P)* is derived from branching points in the polymer (refs. 5,6,8) in the long chain (5A), indicating the occurrence of multiple long chains at branching points.

Fig. 5

In Fig. 5 peaks labeled (a) and (x) are observed in addition to the peaks identified as AMP, PR-AMP, and Ado(P)Rib(P)Rib(P). Peak (x), seen only in the long chain digest, does not have an adenine UV spectrum and no attempt has been made to identify this minor component. Peak (a), observed in both short and long chain digests, does display an adenine UV spectrum and contains radioactivity and, therefore, was clearly derived from the polymer. It is not a degradation product formed during polymer preparation since phosphodiesterase treatment of poly(ADP-ribose) from differing biological preparations gave the same peak (a) components. Furthermore, it is not due to any contaminating

* Ado(P)Rib(P)Rib(P) was identified by nuclear magnetic resonance spectrometry, in agreement with published results (6).

enzymes in phosphodiesterase since treatment of PR-AMP with phosphodiesterase did not produce traces of peak (a). Also, if phosphodiesterase had a phosphatase contamination the amount of peak (a) should be independent of chain length. In view of the arguments that internal branching, and terminal polymer fragments have been accounted for by PR-AMP, Ado(P)Rib(P)Rib(P) and AMP respectively, it is reasonable to postulate that peak (a) is the fragment deriving from the protein attachment site. The existence of such termini has been suggested but no exact chemical structures have been established. We are currently preparing a quantity of peak(a) material for elucidation of its chemical structure by nuclear magnetic resonance spectroscopy, which will be reported elsewhere. It should be noted that we do have some additional chemical evidence bearing on its structure. If PR-AMP is subjected to limited digestion by alkaline phosphatase, a quantity of material is generated which in the HPLC elutes with the same retention time as peak (a). Thus it seems that peak (a) has a structure related to PR-AMP except with a phosphate group absent.

In connection with peak (a) and separation of poly(ADP-ribose) using anion exchange HPLC, it is appropriate to include here an account of additional related studies. If samples of isolated short chain polymer peaks (see Fig. 4A and Table) are subjected to gel electrophoresis, each peak resolves into 2 unequal bands in the gel. Thus it is evident that each polymer peak observed in the HPLC is actually composed of two sub-populations. This finding is consistent with earlier results using hydroxylapatite chromatography (11). We have found that if a single isolated HPLC peak (viz. peak 8) having the two sub-populations is treated with alkaline phosphatase and then re-injected into the HPLC, then two sub-populations are resolved (See Fig. 6A and 6B). Therefore, alkaline phosphatase reacts differently with one sub-population than with the other. It is reasonable to assume that the difference is explained by

Fig. 6

a terminal phosphate group present in only one of the components that is cleavable by the phosphatase. This conclusion presupposes that, before treatment with alkaline phosphatase, the two sub-populations, (differing with respect to presence or absence of a terminal phosphate group) must also differ in some other, compensatory way such as having one less or greater chain length and this results in comigration on the anion exchange resin and co-elution as a single peak.

References

1. T. Sugimura, M. Miwa, H. Saito, Y. Kanai, I. Ikejima, M. Terado, M. Yamada, and T. Utakoji, Adv. Enzyme Regul. Vol. 18 (1980) 195, (Ed. Weber, G. Pergamon Press).

2. E. Kun, T. Minaga E. Kirsten, G. Jackowski, J. McLick, L. Peller, S. M. Oredsson, L. Marton, N. Pattabiraman, and G. E. Milo, Adv. Enzyme Regul. 21 (1983) 177 (Ed. G. Weber, Pergamon Press).

3. T. Minaga and E. Kun, J. Biol. Chem. 258 (1983) 725.

4. T. Minaga and E. Kun, J. Biol. Chem. 258 (1983) 5726.

5. M. Miwa, N. Saikawa, Z. Yamaizumi, S. Nishimura and T. Sugimura, Proc. Nat. Acad. Sci. USA 76, (1979) 595.

6. M. Kanai, M. Miwa, Y. Kuchino and T. Sugimura, J. Biol. Chem. 257, (1982) 6217.

7. H. Juarez-Salinas, V. Levi, E. L. Jacobson, M. K. Jacobson, J. Biol. Chem. 257, (1982) 607.

8. H. Juarez-Salinas, H. Mendoza-Alvarez, V. Levi, M. K. Jacobson and E. L. Jacobson, Anal. Biochem. 131 (1983) 410.

9. J. L. Sims, H. Juarez-Salinas and M. K. Jacobson, Anal. Biochem. 106 (1980) 296.

10. A. Hakam, J. McLick and E. Kun, J. Chromatography 296 (1984) 369.

11. M. Tanaka, M. Miwa, K. Hayashi, K. Kubota, T. Matsushima and T. Sugimura, Biochemistry 16 (1977) 1485.

12. Johnatha B. Crowther, Johom P. Caronia, and Richard A. Hartwick, Anal. Biochem. 124 (1982) 65.

13. M. Kawaichi, K. Ueda and O. Hayaishi, J. Biol. Chem. 256 (1981) 9483.

14. H. Kawamitsu, H. Hoshino, H. Okada, M. Miwa H. Momoi, and T. Sugimura, Biochemistry 23 (1984) 3771.

Legends

Figure 1.
Elution profile of poly(ADP-R) on Sephadex G50 column (column dimensions 93 cm x 1.2 cm). The elution and equilibration buffer was 100 mM Tris-HCl (pH 7.0) containing 1 M NaCl, and fraction volumes were 4 ml, flow rate was 12 ml/hr. Bracket at upper right denotes where mono ADP-R elutes.

Figure 2.
Chain length analysis of unfractionated poly (ADP-R) performed at 75°C monitored by UV detector at 260 nm on AX300 column.

Figure 3.
A. Effect of column temperature on the HPLC pattern of medium chain length poly (ADP-R) from the AX300 column, (a) 29°C, (b) 50°C and (c) 75°C. Equal amounts of medium chain length poly(ADP-R) were injected in the three chromatograms, monitored at 260 nm.

B. Effect of column temperature on the HPLC pattern of long chain length poly(ADP-R) from AX300 column, (a) 29°C, (b) 50°C and (c) 75°C. Equal amounts of polymer were injected and monitored at 260 nm. Note: the broad peak eluted between 80 and 105 min is designated long chain population I and the sharp peak eluting at about 120 min. is designated long chain population II.

Figure 4.
HPLC analysis of (A) short, (B) medium and (C) long chain length poly(ADP-R). Chromatography was performed at 75°C and monitored by UV detector at 260 nm. Selected peaks were UV-scanned at their apexes from 220 to 340 nm, and the spectral plots are shown in the rows of insets. The top row of insets are from (A) (two superimposed in each plot), the middle row from (B) (three superimposed in each plot) and the lower row from (C) (one spectrum in each plot.) In each plot the retention times are given in the upper right hand corner.

Figure 5.
HPLC analysis of phosphodiesterase digestion products of short chain length(A) and long chain length(B), poly(ADP-R) (cf. Fig.1) on AX100 column, performed at 50°C and monitored at 260 nm. Peaks a an x denote unknown components suspected to be terminal groups: b = AMP; c = PR-AMP; d = Ado(P)Rib)Rib(P).

Figure 6.
HPLC analysis of isolated peak 8 from Fig. 4, (a) before and (b) after treatment with alkaline phosphatase. Chromatography was performed on the AX300 column at 75°C and monitored by UV detector at 260 nm.

Table

Compostion of phosphodiesterase digests of ADP-ribose oligomers

Sample	%a	%AMP	%PR-AMP	%Ado(P)Rib(P)Rib(P)	Chain Length
Short Chains:					
Peak 5	8.15	17.38	74.46	---	5.8
Peak 6	6.22	14.35	79.09	0.34	7.1.
Peak 7	5.31	12.51	81.81	0.36	8.2
Peak 8	4.85	11.08	83.65	0.41	9.3
Peak 9	3.98	10.20	85.30	0.46	10.3
Peak 10	3.40	9.59	86.49	0.50	11.0
*Medium:	1.26	6.5.	91.10	1.14	18.6
**Long:	1.79	5.29	89.80	3.02	44.0
Long I:	3.07	4.60	89.95	2.37	44.8
Long II:	1.57	5.21	90.1	3.10	47.4

a = major terminal fragment (see Fig. 5), x in Fig. 5 is not included because it has no adenosine absorbance.

* = incompletely separated medium length fractions were analyzed in one pool.

** Long chains were pooled for analyses.

Fig 1

41.

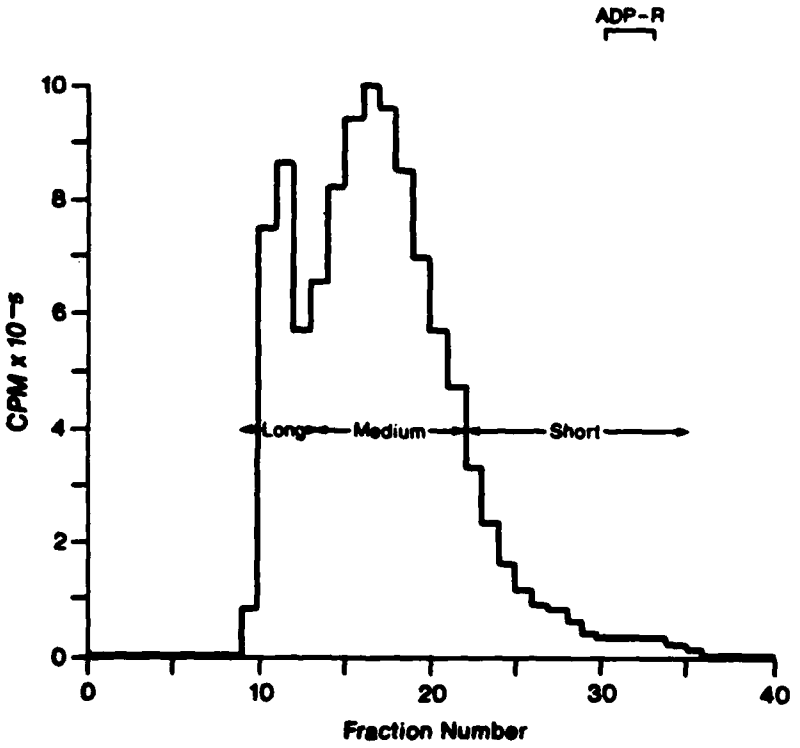
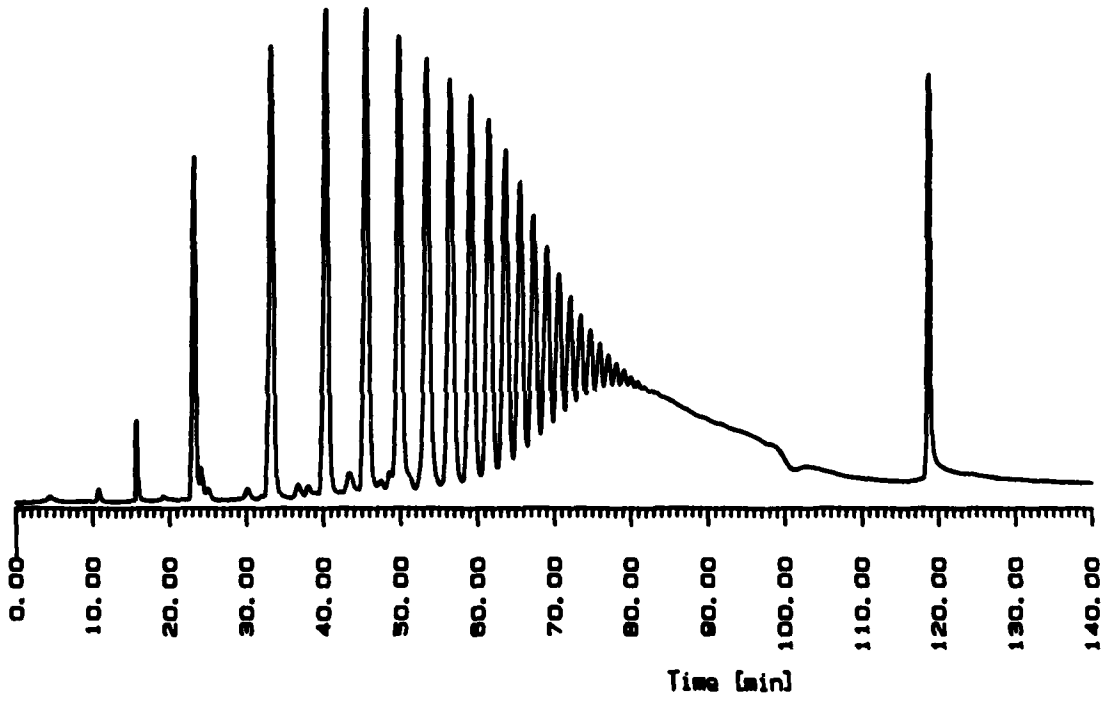
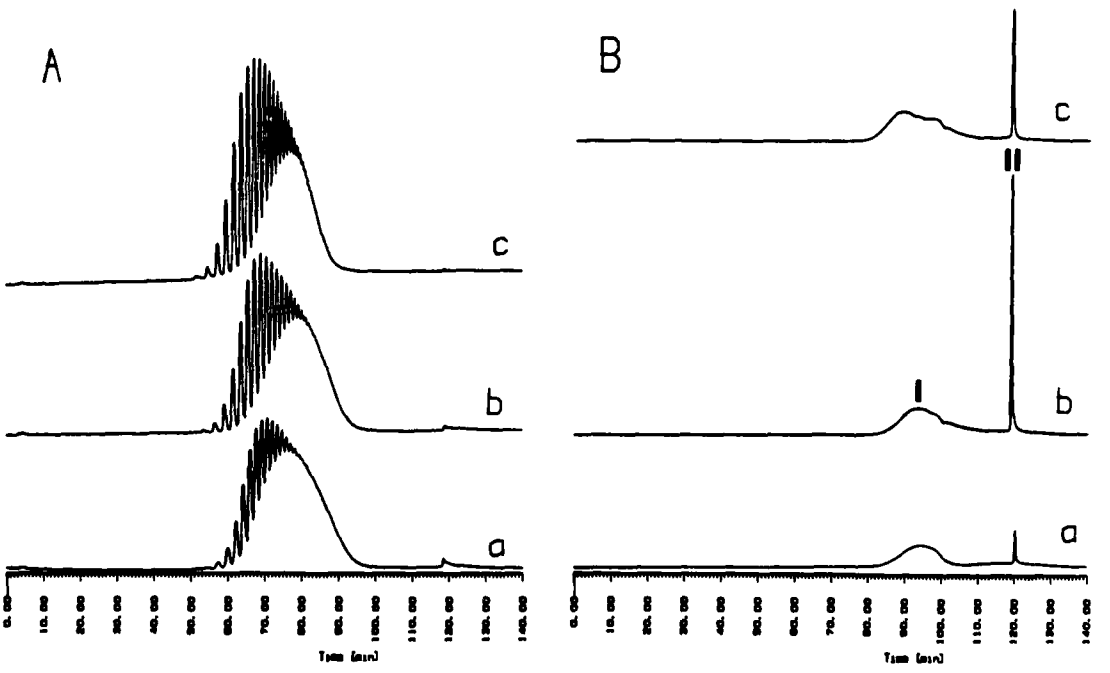
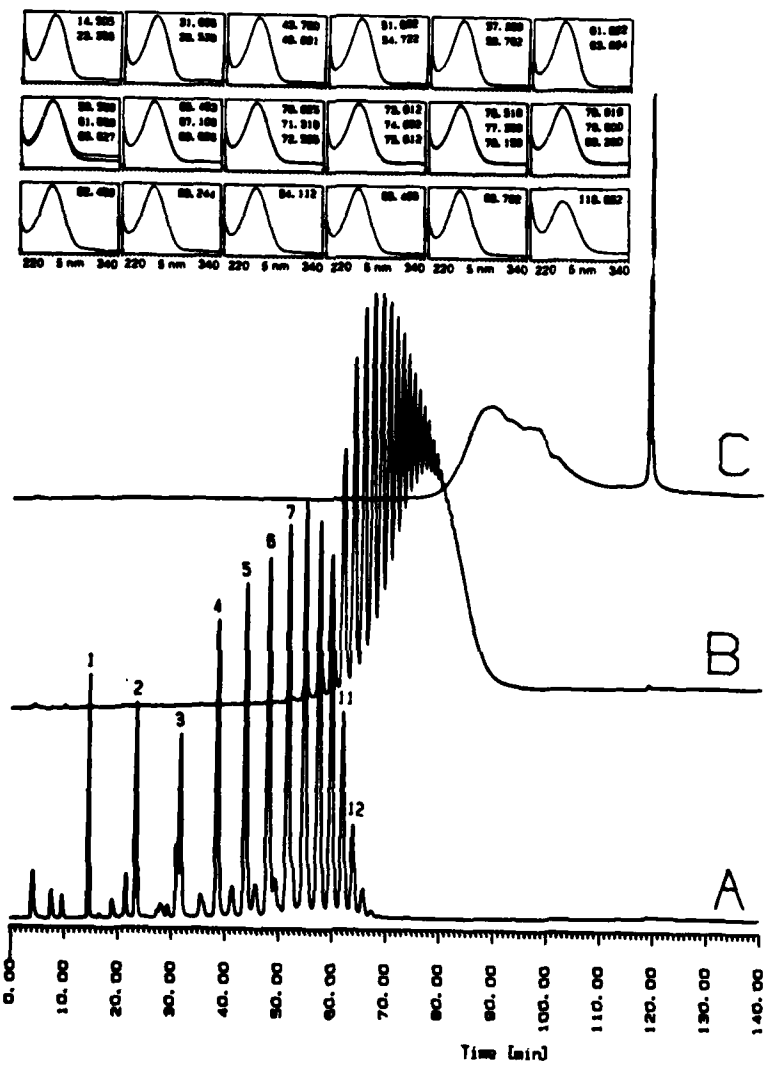
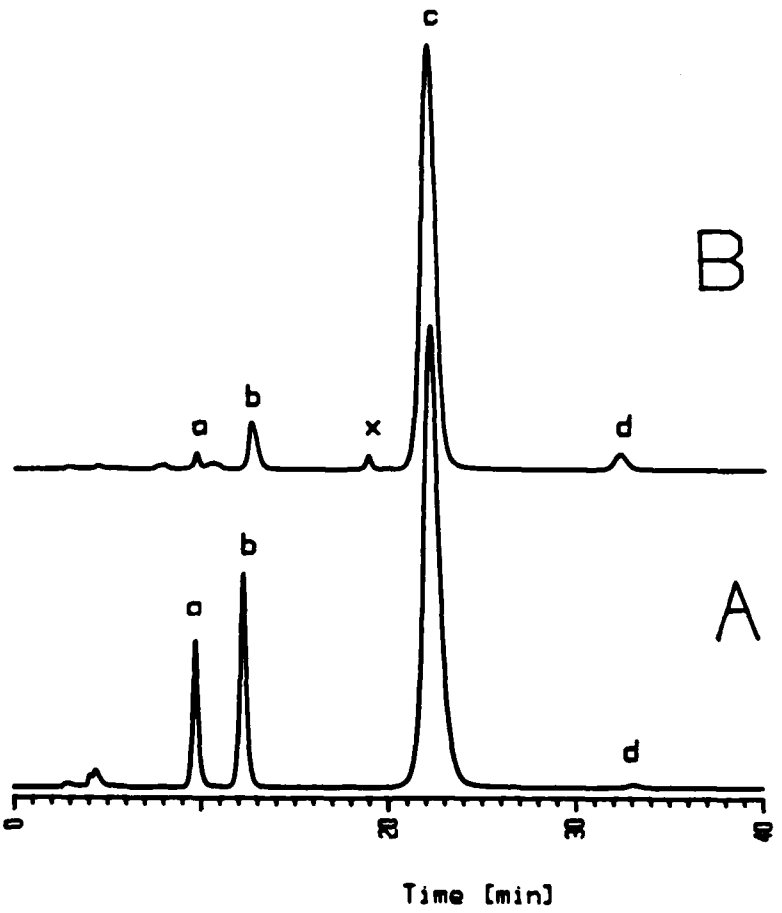

ADP-R
CPM x 10⁻³
10
8
6
4
2
0
Long
Medium
Short
0
10
20
30
40
Fraction Number

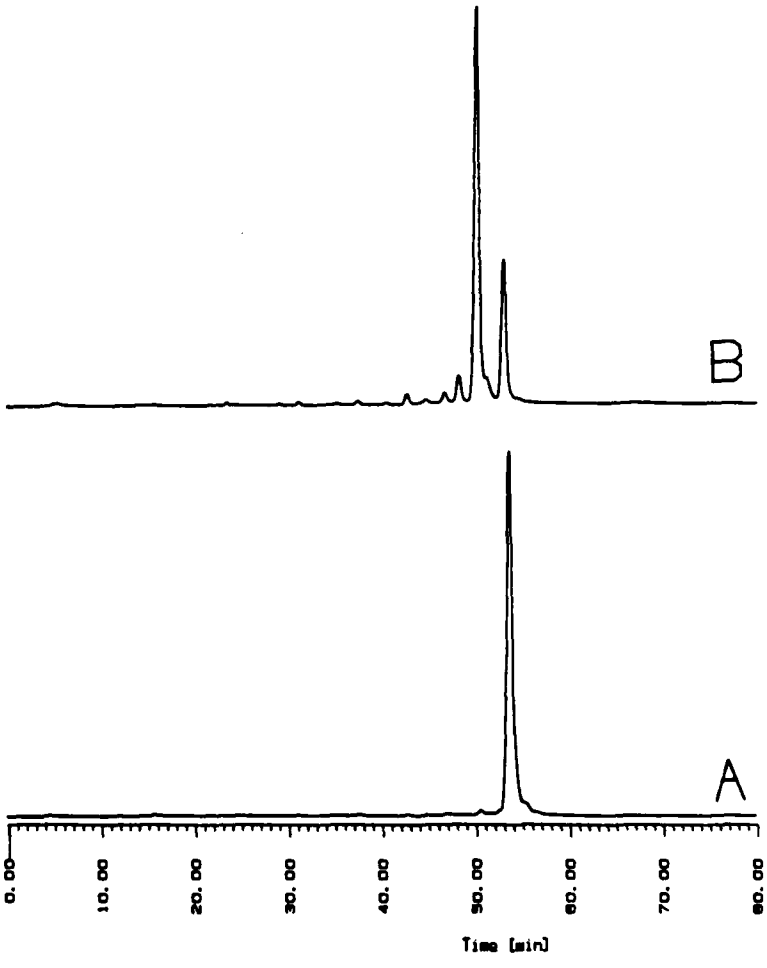
Fig 2


0.00
10.00
20.00
30.00
40.00
50.00
60.00
70.00
80.00
90.00
100.00
110.00
120.00
130.00
140.00
Time [min]


A
B
c
b
a
II
I
Time (min)


220 8 nm 340
C
B
A
1
2
3
4
5
6
7
11
12
0.00
10.00
20.00
30.00
40.00
50.00
60.00
70.00
80.00
90.00
100.00
110.00
120.00
130.00
140.00
Time [min]


c
B
a
b
x
d
b
a
A
d
0
20
40
Time [min]


B
A
0.00
10.00
20.00
30.00
40.00
50.00
60.00
70.00
80.00
Time [min]

3. Mathematical model of polymerization of ADP-ribose.

The mathematical derivations are based on experimental work done on permeabilized 9L-gliosarcoma cells, obtained from cultures (see Fig. 1). Coincidence of certain calculated values (solid line Fig. 2.) and experimental values (dots Fig. 2) indicated that the mathematical model holds for chain elongation, but deviates for initiation. The reasons for this apparent discrepancy may be varying reactivity of initiator sites. The nature of this "variability" of initiator sites is the subject of on-going research.

At the outset, we will assume that there is a single class of initiation sites (I) at which poly (ADP-R) is generated from NAD yielding nicotinamide (NA) as a byproduct. The sequence of steps can be written as:

$$\begin{aligned} &I + NAD \overset{E}{\rightleftharpoons} I\ (ADP\text{-}R) + NA \\ &\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots \\ &I\ (ADP\text{-}R)_{n-1} + NAD \overset{E}{\rightleftharpoons} I\ (ADP\text{-}R)_n + NA \end{aligned} \qquad (1)$$

For a typical step in this chain, a minimal mechanism for the reaction catalyzed by the enzyme poly (ADP-R) polymerase can be suggested.

$$E + NAD \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ENAD \qquad (2a)$$

$$ENAD + I\ (ADP\text{-}R)_{n-1} \underset{k-2}{\overset{k_2}{\rightleftharpoons}} ENAD \cdot I\ (ADP\text{-}R)_{n-1} \qquad (2b)$$

$$ENAD \cdot I\ (ADP\text{-}R)_{n-1} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} ENA \cdot I\ (ADP\text{-}R)_n \qquad (2c)$$

$$ENA \cdot I\ (ADP\text{-}R)_n \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} ENA + I\ (ADP\text{-}R)_n \qquad (2d)$$

$$ENA \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} NA + E \qquad (2e)$$

This scheme involves five steps and ten rate constants; the latter with negative subscripts referring to the depolymerization process. To make the kinetics tractable, these rate constants will be presumed to be independent of the extent of polymerization (n).

A steady state is assumed for the various enzyme substrate complexes in equation (2) and the resulting algebraic equations are solved either by the schematic expansions of the determinants (King and Altman,1956) or by recursive treatment of the difference equations (Bloomfield et al., 1962). The resulting rate expression can be written for $n \geq 1$ ($I\ (ADP\text{-}R)_o$ is then the initiator I).

$$\frac{d\ \{I\ (ADP\text{-}R)_n\}}{dt} = v_f\ \{I\ (ADP\text{-}R)_{n-1}\} - (v_f + v_b)\ \{I\ (ADP\text{-}R)_n\} + v_b\ \{I\ (ADP\text{-}R)_{n+1}\} \qquad (3a)$$

The differential equation for the initiator concentration has a special form:

$$\frac{d\{I\}}{dt} = -v_f\{I\} + v_b\{I\ (ADP\text{-}R)\} \tag{3b}$$

The rates of disappearance of monomer source (NAD) and appearance of by-product (NA) are given by:

$$\frac{-d\{NAD\}}{dt} = \frac{d\{NA\}}{dt} = v_f \sum_{j=0}^{\infty}\{I\ (ADP\text{-}R)_j\} - v_b \sum_{j=1}^{\infty}\{I\ (ADP\text{-}R)_j\}$$

$$= v_f\{I\}_o - v_b \sum_{j=1}^{\infty}\{I\ (ADP\text{-}R)_j\} \tag{3c}$$

The quantities of v_f abd v_b contain the rate constants and concentrations of monomer source {NAD} and side product {NA} in a parametric form dictated by steady state enzyme kinetics i.e.:

$$v_f \equiv \left(\frac{k_2k_3k_4\{E\}_o}{k_{-2}k_4+k_3k_4+k_{-2}k_{-3}}\right)\left(\frac{\{NAD\}/K_1}{1+\{NAD\}/K_1+\{NA\}/K_5}\right)$$

$$= V_f\left(\frac{\{NAD\}/K_1}{1+\{NAD\}/K_1+\{NA\}/K_5}\right) \tag{4a}$$

and

$$v_b \equiv \left(\frac{k_{-2}k_{-3}k_{-4}\{E\}_o}{k_{-2}k_4+k_3k_4+k_{-2}k_{-3}}\right)\left(\frac{\{NA\}/K_5}{1+\{NAD\}/K_1+\{NA\}/K_5}\right)$$

$$= V_b\left(\frac{\{NA\}/K_5}{1+\{NAD\}/K_1+\{NA\}/K_5}\right) \tag{4b}$$

V_f anf V_b are maximum velocities in the customary Michaelis-Menten sense and K_1 and K_5 are dissociation constants of nicotinamide dinucleotide and nicotinamide respectively from the enzyme. The fact that dissociation constants rather than more complex aggregates of the rate constants occur here is a consequence of the simplified reaction scheme in (2) and the requirements that $\{I\}_o \ll \{E\}_o \ll \{NAD\}_o$ and {NA}. A more elaborate mechanism would lead to different and more complex representations for v_f and v_b but with the form of equations (3a) - (3c) unaltered.

In the early stages of the reactions before there is an appreciable accumulation of by-product or if the reaction is sufficiently irreversible, the terms involving v_b can be neglected. The resultant set of differential equations is identical to that initially proposed for the alcohol initiated polymerization of ethylene oxide(Flory,1940). They are known to yield as a solution for the weight fraction of polymeric product of chain length

(n) $W_n \equiv n \{I (ADP\text{-}R)_n\} / \sum_{j=0}^{\infty} \{ (ADP\text{-}R)_j \}$ close to a Poisson distribution i.e.

$$W_n \cong \nu^n (\exp -\nu)/n! \tag{5a}$$

where

$$\nu \equiv \langle n \rangle = \int_o^t v_f dt = \{(NAD)_o - (NAD)/(I)_o\} \tag{5b}$$

The quantity ν is the number average degree of polymerization and all the particular features of the reaction are contained in the expression.

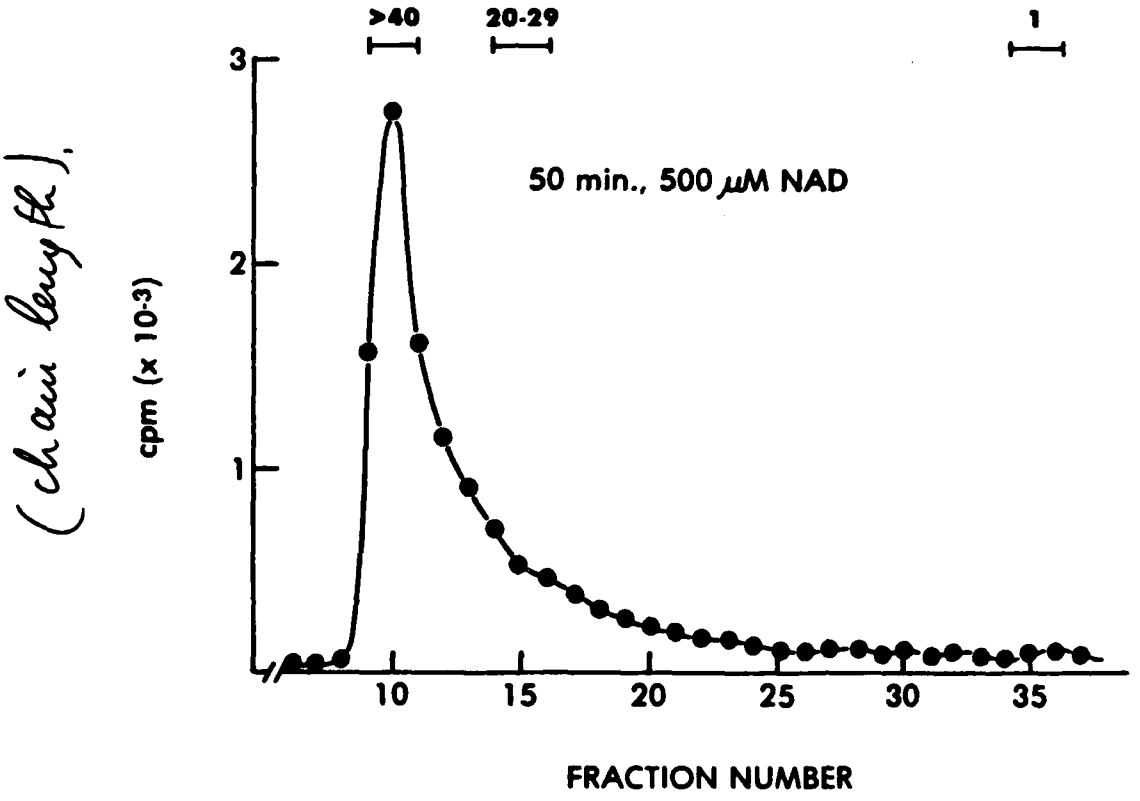
It facilitates calculation of the expected product distribution to express the factorial in equation (5a) by the extended Stirling's approximation (Margenau and Murphy, 1943) viz $n! = n^n e^{-n} \sqrt{2\pi n}$. W_n is a narrow distribution (Flory,1940; Gold,1958) with a maximum at $n \approx \nu$ and a value for $W_\nu = 1/\sqrt{2\pi\nu}$. Referring the weight fractions at all species to the maximum leads to the result that

$$W_n/W_\nu = (\nu/n)^{n+\frac{1}{2}} \exp (n-\nu) \tag{6}$$

which is also sharply peaked with, of course, a maximum equal to unity at $n = \nu$.

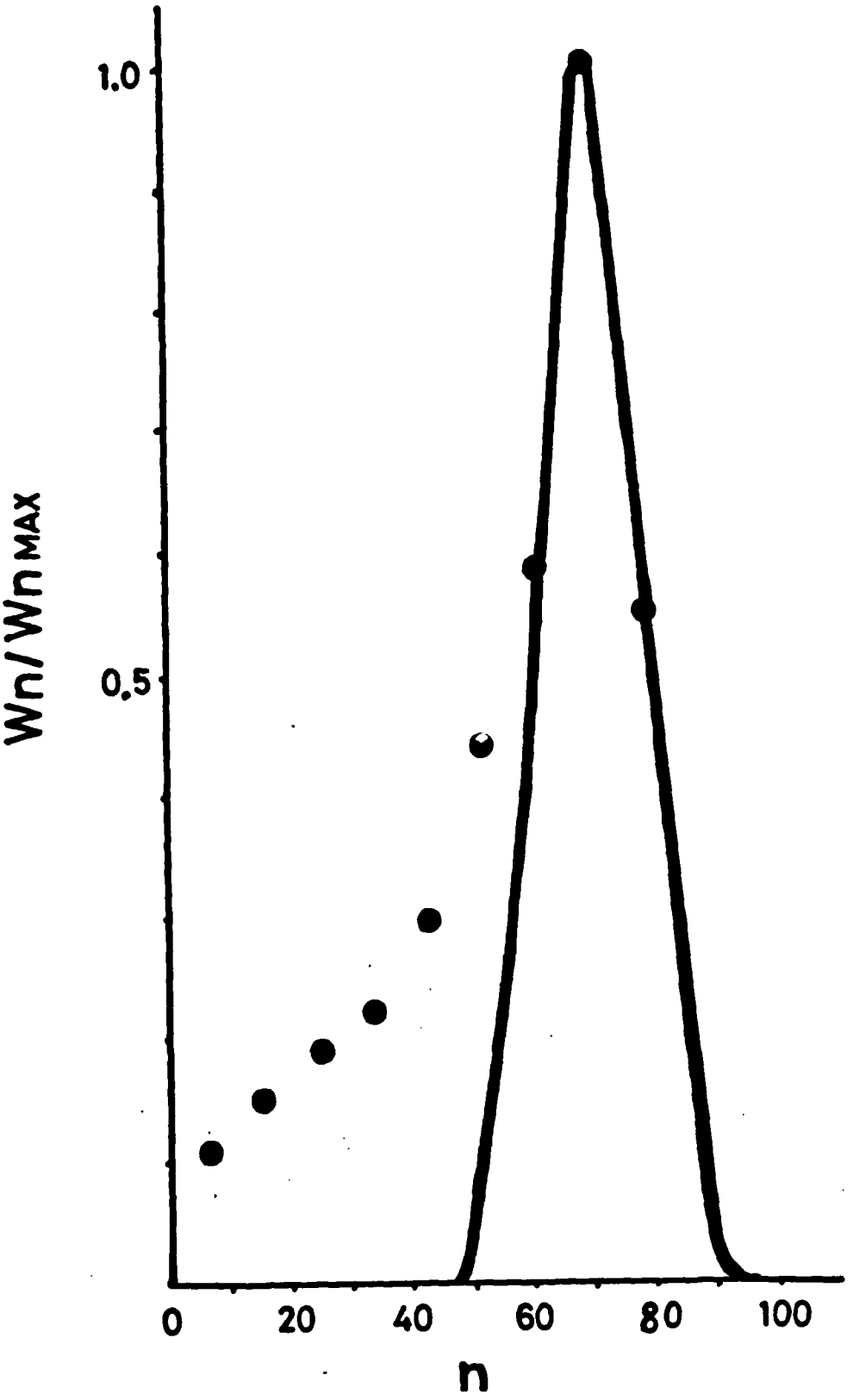
Fig 1.

Seize distribution of in vitro synthesized $(ADP\text{-}R)_n$



(chain length).

Fig 2.



II. Biological and cell structural studies of poly ADP-ribosylation in cellular and sub-cellular systems.

(Summary)

The *in vitro* rates (v_{init}) of poly(ADP-ribose) polymerase of permeabilized rat hepatocytes and of nuclei, isolated from hepatocytes, did not differ significantly. Incubation beyond 3 minutes resulted in diminished poly ADP-ribosylation in hepatocytes compared to nuclei, coinciding with high rates of plasma membrane associates NAD-glycohydrolase. Cultured cells (Drosophila K_c cells, gliosarcoma 9L, human fibroblasts and mouse spleen lymphocytes) exhibit variations of NAD-glycohydrolase and poly(ADP-ribse) polymerase activities and the assessment of poly(ADP-ribose) polymerase activity in permeabilitzed cells requires simultaneous assay of NAD-glycohydrolase. In rat liver nuclei during 10 min. incubation with 500 μM NAD, 40% of NAD is consumed, 10% ADP-ribose is bound to proteins, and 20% ADP-ribose, 5% AMP and 2.7% adenosine are liberated. As determined by solvent partitioning (Jackowski, G. and Kun, E., J. Biol. Chem. 258, 1983, 12587,) the phenol soluble protein-ADP-ribose fraction represents largely mono-ADP-ribose protein adducts whereas the H_2O soluble phase contains poly ADP-ribosylated proteins. The quantity of ADP-ribose protein adducts, the chain length of oligomers and the nature of apparent acceptor proteins in liver nuclei vary significantly with the concentration of NAD as substrate. At 500 μM NAD concentration the quantity of ADP-ribose containing adducts was in the n mol per mg DNA range, the polymers are long chains and the acceptor proteins predominantly non-histone proteins. At 0.1 μM NAD as sustrate pmol quantities of monomeric ADP-ribose adducts per mgDNA were formed and the main acceptors were sharply discernable on the basis of molecular mass as histones, high mobility nonhistone proteins, two protein groups of a mass of 66 and 44 kDa respectively, and the poly(ADP-ribose) polymerase enzyme protein of 119 kDa mass. Whereas products in the presence of 0.1μM NAD may indicate acceptors of highest reactivity, protein adducts formed in the presence of 500μNAD resemble a pattern found *in vivo*.

We have previously shown that mono and poly(ADP-ribose) adducts of nuclear proteins of cardiocytes are quantitatively separable by a method of phenol-H_2O partitioning followed by gel electrophoresis (1). This experimental technique is likely to be applicable to a variety of cell types and may be useful for the study of the cell biological importance of the covalent modification of nuclear proteins by poly ADP-ribosylation, (2,3,4,5,6).

The present work is concerned with the nature of mono and poly(ADP-ribose) protein adducts in liver tissue. Availability of surviving hepatocytes (7,8) provided a model for comparison of hepatocytes with several types of cultured cells, e.g. Drosophila K_c (9,10), gliosarcoma 9-L (11), human fibroblasts (12) and mouse spleen lymphocytes (13), and we determined the interference of the cell wall located NAD-glycohydrolase with the enzymatic assay of nuclear poly ADP-ribosylation in permeabilized cells. Enzymatic rates were studied in hepatocytes and nuclei isolated from hepatocytes in order to evaluate the presumed latency of poly(ADP-ribose) polymerase in cells (14). We also compared in isolated liver nuclei the effects of near physiological concentration of NAD (500μM) with a very low concentration of NAD (0.1μM) as substrates on the nature and quantity of ADP-ribosylated proteins synthesized in vitro with the intention to identify the most reactive ADP-ribose acceptor proteins.

Experimental Procedures

1. Hepatocyte monolayer cultures were prepare from livers of fed female rats of the Wistar strain after perfusion and disintegration of the interstitium of liver with collagenase (15) dissolved in hemoglobin-free medium (7, 8) as follows. Rats (180-200 g body weight) were anesthetized with nembutal, livers removed and perfused under aseptic conditions with 25 mg collagenase/100 ml medium for 25 min (37°), then the cells, obtained by opening the liver capsule and gentle

disintegration of the liver tissue with a glass rod, were suspended in Krebs-Henseleit solution (containing 5.5 mM glucose) and filtered through nylon (75μ mesh). After centrifugal washing (twice at 15 x g for 2 min.) liver cells were suspended in a modified Waymouth (W/AB 77) medium (cf. 7,8) supplemented with 10% fetal calf serum, 1 μM insulin, streptomycin (50μg/ml) and penicillin (50 μg/ml). The cellular yield was about 50% and viability greater than 90% as determined by the trypan blue exclusion technique (cf. 7, 8). Aliquots of 25 to 50 mg liver cells, were dispensed in Falcon Petri dishes (60-100 mm diameter) precoated with collagen, previously sterilized by UV radiation. Collagen was isolated from rat tails (16). The dispersed cells were incubated in 95% air, 5% CO_2 atmosphere at 37°. Hepatocytes were allowed to attach to the collagen for 3 h and the suspending medium, containing also non-attached debris and other cell types, was quantitatively removed by aspiration. The homogenous hepatocyte population attached to collagen was incubated with fresh medium for various lengths of time (6 h to 72 h), then eluted from the dishes by washing with 0.9% NaCl, subsequently frozen (-80°) and stored either for enzyme assays after permeabilization or for the isolation of nuclei.

2. Cultures of Drosophila K_c cells (9, 10), gliosarcoma 9-L cells (11) and human fibroblasts (12) were grown by published procedures. 9-L gliosarcoma cells were a gift from Drs. Laurence Marton and Burt Feuerstein (Brain Tumor Research Center, UCSF) and mouse spleen lymphocytes from Dr. Dan Stites (13). Cell pellets, obtained from cultures, were kept at -80°C in aliquots of 5×10^6 cells per pellet and only thawed once for cell permeabilization and enzymatic assays. Enzymatic rates in stored cells were indistinguishable from those obtained with freshly harvested and permeabilized cells. Two methods of cell permeabilization were employed (17, 18) yielding indistinguishable results.

3. Assay systems for poly(ADP-ribose) polymerase for permeabilized cells (19) and for nuclei (1) have been described and the test system for NAD-glyco-

hydrolase was the same as reported (20). DNA was determined fluorometrically (21) or colorimetrically (cf 1.) and protein by spectrophotometry (22). Liver nuclei were isolated by a published method (23) except Triton X-100 was omitted from the isolation medium. The same techique was employed for the isolation of nuclei from cultured cells, except batches of approximately 60 x 10^6 cells in 2 ml of 0.35M sucrose (pH 7.2) were first disrupted in a Dounce homogenizer (at 0°) by about 6 to 10 strokes. Cell disruption was controlled by microscopic examjnation. After incubation of permeabilized cells or nuclei at 25° for varying periods (see Results), the reaction was stopped with 1 ml 20% $HClO_4$ (0° ice bath for 10 min.) and the centrifugal sediment (100 x g for 10 min. at 4°C) washed 3 times with 5 ml 10% $HClO_4$, then the isolation procedure of protein adducts was continued as follows.

Quanitative separation and isolation of poly ADP-ribosylated proteins by solvent partitioning was done by an abridged modification of the previously described technique (1). The protein pellet was suspended in 1 ml solubilizing buffer [20 mM EDTA, 20 mM Tris-HCl, pH 6.5, 0.1% 2-mercaptoethanol, 0.1 mM phenyl methyl sulfonyl fluoride (PMSF) and 2%SDS] and dissolution of the pellet completed by mechanical stirring at 10°C for 60 min. These conditions were found to preserve polymer-protein bonds (1). Extraction of phenol soluble components was done by vigorous stirring for 2 hours at 10° with 1 ml phenol (saturated with the above solubilizing buffer from which SDS had been deleted). The aqueous and phenol phases were quantitatively withdrawn and the aqueous phase combined with the interphase. Labeled protein-polymer adducts present in the fractions extracted from hepatocyte nuclei were determined by isotope analysis at this stage, whereas products of the whole liver nuclei were processed as follows. The phenol fraction was extensively dialyzed at 10°C against 200 volumes of electrophoresis buffer of pH 6.5(cf. 1) in Spectrapor

tubing (mol. wt. cut off 12 to 14 x 10^3) for 12 hours with 3 successive changes of the buffer. In experiments where instead of 500 μM NAD the concentration was 0.1 μM, dialysis tubing with a cut-off of 10^3 was employed. DNA in the aqueous phase was removed by digestion with DNA-ase I (1). The volume of dialysate was reduced to desired size by Sephadex G-50 (coarse grade). Proteins from both fractions were precipitated by 10% $HClO_4$ for electrophoretic or other types of analyses (1). Determination of polymer chain length, Cs_2SO_4 density gradient ultracentrifugation and gel electrophoresis were carried out as reported (1). Quantitative analyses of NAD and its 10% $HClO_4$ soluble catabolic products were carried out by HPLC in a phosphate-methanol concave gradient on a reverse phase column (Ultrasphere ODS, 5μ Altex-Beckman) on a Waters Model 680 Gradient Controller as reported (24).

RESULTS

Initial rates of protein poly ADP-ribosylation by hepatocytes and isolated hepatocyte nuclei are shown in Figure 1. Calculation of these rates was based on DNA content, therefore they represent the activity per nuclei. The slight discrepancies between v_{init} of cells vs. nuclei within the first 1 to 3 minutes is within experimental variations ($\pm$ 15%), therefore initial rates of poly ADP-ribosylation are indistinguishable in permeabilized cells or in isolated nuclei. However, when rates are followed beyond 4 minutes, isolated nuclei tend to exhibit somewhat elevated rates before both types of reactions reach a plateau.

Figure 1 here

A plausible explanation for this time dependent discrepancy between whole cells and nuclei is an artefactual alteration of apparent protein-poly ADP-ribosylation rates in whole cells by reactions that are independent from poly(ADP-ribose) polymerase. The most important emzymatic process that can falsify apparent poly(ADP-ribose) polymerase rates in whole cells are cell wall-associated NAD-glycohydrolase activities (Table I). The activity of this enzyme in isolated hepatocyte nuclei is below the detection limit of the assay method (not shown) but can be very high in whole cells especially in hepatocytes (Table I, No.5). The plasma membrane associated NAD-glycohydrolase can introduce artefacts that are unrelated to nuclear poly(ADP-ribose) polymerase by the accumulation of nicotinamide that progressively inhibits the polymerase and by a depletion of added NAD, provided the NAD glycohydrolase activity is much greater (see Table I, No.5) than that of poly(ADP-ribose) polymerase. As would be anticipated, this artefact is significant upon prolonged incubation (Fig.1). Rates of

Table I here

nicotinamide release from NAD by permeabilized cells vary greatly, depending on the cell type, and only the Drosophila K_c cell shows exact stoichiometry between rates of nicotinamide release and ADP-ribose-protein binding, indicating the absence of extra glycohydrolase activity (Table I, No. 1). Inhibition of both reactions in Drosophila cells by benzamide supports this conclusion, whereas in all other cell types, significant discrepancies are apparent. A variable but significant inhibition of nicotinamide release by benzamide was observed in certain cell types (No. 2, 4 Table I). Since plasma membrane or mitochondrial outer-membrane associated NAD-glycohydrolases were insensitive to benzamide, (Kirsten, E. and Kun, E, unpublished), it seems likely that nuclear poly (ADP-ribose) polymerase in certain cell types may significantly hydrolyze NAD beyond the rate that is stoichiometric with ADP-ribose-protein formation (cf. 3,4). It is known that poly(ADP-ribose) polymerase possesses intrinsic NAD-glycohydrolase activity (26) and this may vary in certain cell types.

Mono and poly(ADP-ribose protein adducts are separable by solvent partitioning (1). We compared the distribution of total <u>in vitro</u> generated protein adducts in permeabilized hepatocytes and nuclei isolated from hepatocytes. Results expressed as p mol ADP-ribose protein adducts per μg DNA present in the phenol and aqueous phases (5.6 in cells and 5.9 in nuclei in the phenol fraction, and 14.4 and 16.1 in the aqueous phase per cells and nuclei respectively) demonstrate that no large differences could be found between isolated nuclei and permeabilized cells, except an apparent 20% discrepancy after 15 min. incubation. This can be explained by the activity of the cell membranes associates glycohydrolase (compare with Fig. 1).

Table II here

The chromatographic analyses of acid soluble products following incubation of liver nuclei with 500 μM NAD for 10 min. is given in Table II. At the end of the incubation 60% of NAD remains in the system, whereas the main catabolic product is ADP-ribose, indicating the activity of poly(ADP-ribose) glycohydrolase that exceeds twice the activity of poly-(ADP-ribose) polymerase. This is in variance with hepatocyte nuclei which have almost undetectable NAD-glycohydrolase activity (see above). Some degree of pyrophosphorolytic degradation of ADP-ribose to AMP and adenosine is also apparent, however the absence of phosphoribosyl-AMP (24) seems to exclude pyrophosphorolytic degradation of the polymer itself, even though a pyrophosphatase that performs this reaction has been reported (25). According to results shown in Table II, about 9-10% of NAD is converted to protein-poly(ADP-ribose) adducts within the relatively short period of incubation.

The distribution of poly ADP-ribosylated proteins generated by liver nuclei was determined under previously standardized conditions in the presence of 500 μM NAD (1) in 8 separate experiments, and 75.6±6%SD, $n = 8$) of polymer protein adducts were found in the aqueous phase indicating that two-thirds of adducts contain long chain polymers, which is significantly different from results obtained with cardiocyte nuclei, where more than 90% of adducts were present in the aqueous phase (1). The presumably cell type specific differences of distribution of protein-polymer adducts in the phenol and aqueous fraction, prepared from relatively uniform nuclei of cardiocytes (1) and from liver nuclei derived from a mixture of hepatic cell types, are difficult to evaluate at present and will be more meaningful as soon as various isolated liver cell types will become available for biochemical studies. Analyses (1) of enzymatic reaction products that were formed by

isolated liver nuclei in the presence of 500 μM NAD during an incubation of 10 min. duration gave the following results. The chain length of polymers present in the phenol and aqueous fraction is shown in Fig.2. As with cardiocyte nuclei (1) the phenol phase contained predominantly monomeric ADP-ribose protein adducts with some oligomers, whereas long chain poly-(ADP-ribose)-protein adducts separated into the aqueous fraction, indicating their "nucleic-acid"-like solubility.

Figure 2 here

In order to ascertain that the extracted polymer protein adducts are not a fortuitous mixture of proteins and possibly free polymers we subjected adducts present in both aqueous and phenol phases to Cs_2SO_4 density ultra-centrifugation (1) and results are illustrated in Fig.3. The top part (I) shows that poly(ADP-ribose) associated radioactive material separates by density characteristics of protein adducts in both aqueous (A) and phenol phases (B) and in part II of Figure 3 base hydrolysis of polymer protein adducts from both phases (C) results in a sedimentation characteristic of radioactive material that is identical with that of free poly(ADP-ribose) (D) (1).

Figure 3 here

The quantity and nature of acceptor proteins were determined under two distinct experimental conditions, defined by the concentration of added NAD as substrate. We have in the past as well as in present experiments routinely used 500 μM NAD (labeled with ^{14}C in the adenine moiety, with a specific radioactivity in the range of 20,000 d.p.m./n mol) which allows the detection of the order of 30-50 n mol protein bound ADP-ribose per mg DNA. Gel electrophoretic separation(1) of adducts present in the aqueous phase (75%) and in the phenol phase (25%)

Figure 5 goes here

is demonstrated in Fig. 5. Clearly the proteins migrating in the mol. wt. range of the histone and high mobility non-histone protein group serve prominently as ADP-ribose acceptors. Upon incubation for 10 minutes a protein peak, exhibiting the approximate electrophoretic migration properties of poly(ADP-ribose) polymerase (ca. 116 kDa), becomes strongly labeled, besides proteins of a mass of 66 and 45 kDa. It is of importance that only 6-10% of ADP-ribose from added NAD appeared as protein adduct in both experiments shown in Figs 4 and 5. Furthermore, the quantities of ADP-ribose protein adducts in Fig. 5 belonging to the phenol soluble group, are in the order of p mol/mg DNA, therefore could not have been detected with ^{14}C-labelled NAD of much lower specific radioactivity (compare with Fig. 4).

Protein - mono ADP-ribose adduct formation at 0.1μM NAD as substrate (Fig. 5) was inhibited by 100μM benzamide, a known inhibitor of poly(ADP-ribose) polymerase (27). Therefore, these monomeric ADP-ribose adducts in all probability represent poly(ADP-ribose) polymerase catalyzed reaction products.

11.

DISCUSSION

Our results (Fig.1) do not indicate a substantial latency of poly(ADP-ribose) polymerase in hepatocytes as determined by initial velocity kinetics of permeabilized cells and cell nuclei. Variance with reported results (14) is likely to be due to differing experimental conditions, most significantly by prolonged reaction time (14). As we show (Fig. 1), beyond 1-2 minutes interference by cell membrane NAD-glycohydrolase on the apparent rates of poly(ADP-ribose) polymerase becomes significant. Determination of poly(ADP-ribose) polymerase activity of permeabilized cells critically depends on factors illustrated in Table I., and our results show that assays of poly(ADP-ribose) polymerase activity in permeabilized cells can be evaluated only if cell membrane NAD-glycohydrolase is simultaneously determined. Our results agree with those obtained with perfused liver (28,29) demonstrating high rates of hydrolytic degradation of external NAD by plasma membrane associated enzymes. These enzymes do not participate in the intracellular catabolism of NAD, that primarily depends on poly(ADP-ribose) polymerase (30).

Evidence for the physiological activity of poly(ADP-ribose) polymerase in animal cells is their polymer content. It is of interest to compare <u>in vivo</u> analytical values with rates of syntheses of polymer protein adduct <u>in vitro</u>. The larger than tetrameric polymer in rat liver corresponds to about 1 n mol ADP-ribose adduct/mg DNA as determined by an immunological assay (31). By affinity chromatography, that does not discriminate between short and long chain polymers (32), hamster liver polymer content was found to be 15 n mol/mg DNA, in good agreement with 12.5 n mol/mg DNA ADP-ribosylated protein content of rat liver, obtained by entirely different techniques (33). If <u>in vitro</u> polymerization by liver nuclei in the presence of 100 to 500 μM NAD as substrate

is allowed to plateau, the total quantity of protein bound ADP-ribose is consistently on the average 20 to 50 n mol/ mg DNA representing maximal values, a roughly similar order of magnitude as found in normal liver in vivo.

In vivo labelling kinetics of poly(ADP-ribose) with [^{14}C]-ribose also indicate a significant turnover of the polymer in intact cells (32, 34), that is probably regulated by as yet incompletely understood mechanisms (cf. 35). Results shown in this paper suggest that these processes can be studied in the systems described, provided possible side reactions of NAD catabolism are accounted for.

The initial velocity protein labelling pattern with ADP-ribose at 0.1 μM NAD significantly differs from apparent steady states obtained in vitro with 500 μM NAD as substrate(compare Fig. 4 and 5). Since products with 0.1 μM NAD are all monomeric adducts, yet sensitive to inhibition by benzamide, our tentative interpretation of these results is that initiator sites of poly (ADP-ribose) polymerase catalyzed reactions have been determined. Similar results were obtained by others employing low concentrations of NAD in vitro or by in vivo labelling (36, 37, 38). It is of importance to note that the kinetics of protein poly ADP-ribosylation only partially follows a Michaelis-Menten Model, elongation beyond short oligomers is in accord with the chemical kinetics of polymerization (cf. 6). It is also known that apparent K_m values for NAD vary with the concentration of coenzymic DNA (41). For these reasons the monomeric adducts of selected ADPribose proteins (Fig. 5), synthesized under V_{init} conditions may represent iniatiator sites regulated by structural factors in chromatin which are as yet unknown. The projection of these results to intact cellular condition requires further studies. In vivo, as well as under steady

state conditions in vitro in the presence of 500 μM NAD, mechanisms of protein ADP-ribosylations in nuclei are complicated by the simultaneous participation of several reactions, besides poly(ADP-ribose) polymerase. The histone or casein mono(ADP-ribose) transferase (39) as well as the chemical addition of ADP-ribose to nuclear acceptors (40) both can synthesize templates for subsequent polymerization (26.) Identification of either of these reactions as rate limiting in the overall process of poly ADP-ribosylation has thus far not been carried out .

LEGENDS

Figure 1. Hepatocytes, cultured for 6 h and permeabilized either by lysolecithin (17) or by hypotonic treatment (18), or nuclei isolated from hepatocytes, equivalent to 2 µg DNA, were incubated at 25°C in a total volume of 25 µl reaction mixture composed of 100 mM Tris-Cl, pH 8.5, 2.5 mM dithiothreitol, 10 mM EDTA, 20 mM $CaCl_2$, 0.5 mM NAD, ^{14}C-labeled (adenine moiety, 40,000 dpm/n mol) and 0.25 mM PMSF. The reaction was started by the addition of cells or nuclei in a volume of 10µl, and terminated at given time intervals by addition of 10% $HClO_4$; acid precipitable radioactivity was determined as described (35). n=5

Table I. Protein bound poly(ADP-ribose) formation was assayed as described in legend to Fig. 1 and NAD glycohydrolase as follows. Lysolecithin-permeabilized cells were incubated at 25° as described in the legend to Fig. 1, except that NAD was labeled in the carbonyl moiety (20,000 dpm/n mol). The reaction was terminated by addition of 200 µl 10% trichloroacetic acid and released nicotinamide was determined as published (20).

Figure 2. The analysis of polymer chain length following alkaline hydrolysis of adducts after solvent partitioning (1) is shown. Comparable quantities (cpm) of aqueous and phenol soluble polymer protein adducts were subjected to molecular filtration on Sephadex G-25.

and authentic polymers and oligomers served as chain length standards (N_{av} in Fig.2). The phenol phase contains mono and oligomeric protein adducts, whereas medium and long chain polymers are present in the aqueous fraction. n=3.

Figure 3. Cs_2SO_4 density ultracentrifugation (12,15) of poly(ADP-ribose) protein adducts present in the aqueous (A,C) and phenol soluble (B) phases and after base hydrolysis (D). Top of the gradient = right end of abscissa. m = 2

Figure 4. Gel electrophoretic separation (1%) of poly(ADP-ribose) and oligo-ADP-ribose protein adducts formed in the presence of 500 μM NAD in both H_2O soluble (Aqueous,A) and phenol soluble (Phenol,B) fractions. Solid lines = radioactivity; dotted lines = A_{570} of Coomassie stained protein. The quantity of ADP-ribose-protein product shown in Fig.4 is equivalent to 0.5 n mol/10^4 cpm, which is 100 n mol/mg DNA per 10 min. incubation. n = 3

Figure 5. Gel electrophoretic separation of phenol soluble ADP-R protein adducts formed upon incubation of rat liver with 0.1 μM NAD. Incubations were performed as described (1), except NAD was 0.1 μM, ^{32}P-labeled in the adenylate moiety (approx. 600,000 dpm/p mol). ADP-ribose-protein adducts were precipitated with 20% perchloric acid; following centrifugal washings, solubilization and phenol partitioning was performed as described in Experimental Procedures. The total amount of adducts formed was 3 and 10 p mol/mg DNA, in the 30 second and in 10 minutes, respectively. In both cases 85% of the total adducts partitioned into the phenol phase. After dialysis aliquots of the phenol soluble fractions (approximately 40 μg protein) were subjected to electrophoresis (1). After electrophoresis the gels were cut into 2.5 mm slices, digested in 2% periodic acid, and radioactivity measured by scintillation counting. The dotted line represents the adducts formed in the 30 second incubation, the solid line those of the 10 minute incubation. n=3

References

1. Jackowski, G. and Kun, E., J. biol. chem. 258 (1983) 12587

2. Hilz, H. and Stone, P.R., Rev. biochem. pharmacol. 76 (1976) 1

3. Hayaishi, O., and Ueda, K., Ann. rev. biochem. 46 (1977) 95

4. Hayaishi, O., and Ueda, K. (Eds.)"ADP-ribosylation reactions, biology and medicine" (mol. biol. series, Acad. Press, N.Y. (1982)

5. Pekala, P.H., and Moss, J. "Curr. top. cell. regul. 22 (1983) 1

6. Kun, E., Minaga, T., Kirsten, E., Jackowski, G., McLick, Jr., Peller, L., Oredsson, S.M., Marton, L., Pattabiraman, N., and Milo, G.E., Adv. enzyme regul. 21 (1983) 177

7. Bischoff, E., Wildening, J.., Tan-Thi Ti-A., and Decker, K., Eur. j. biochem. 62 (1976) 279

8. Seglen, P., Exptl. cell res. 82 (1973) 391

9. Stevens, B., Alvarez, C.M., Bohman, R. and O'Connor, J.D., Cell 22 (1980) 675

10. Savakis, C., Demetri, G., and Cherbas, P. Cell 22 (1980) 665

11. Baker, M., Hashino, T., Gurcay, O., Wilson, C.B., Nielson, S.L., Downie, R., and Eliason, J., cancer res. 33 (1973) 976

12. Milo, G.E., and DiPaolo, J.A., Nature, 275 (1978) 130

13. Pavia, C.S. and Stites, D. P., J. immunol. 23 (1979) 2194

14. Althaus, F.R., Lawrence, S.D., Suttler, G.L., and Pitot, H.C., J. biol. chem. 257 (1982) 5528

15. Berry, M.N. and Friend, D.S., J. cell. biol. 43 (1969) 506

16. Michalopoulos, G. and Pitot, H.C., Exptl. cell. res. 94, (1973) 70

17. Miller, M.R., Castellot, J.J. and Pardee, A.B., Biochemistry, 17 (1979) 1073

18. Halldorsson, H., Gray, D.A. and Shall, S., FEBS lett. 85 (1978) 349

19. Kun, E., Kirsten, E., Milo, G.E., Kurian, P. and Kumari, H.L., Proc. natl. acad. sci. USA 80 (1983) 7219

20. Ueda, K., Fukushima, M., Okayama, H., Hayaishi, O. (1975) J. biol. chem. 250 (1975) 7541

21. Setaro, F. and Morley, C. Anal. biochem. 71, (1976) 313

22. Lowry. O.H., Rosebrough, N.J., Farr, A.L. and Randall, R. J., J. biol. chem. 193 (1951) 265

23. Liew, C.C. and Chan, P.K. Proc. natl. acad. sci. USA 76, (1976) 3458

24. Hakam, A., McLick, J. and Kun, E. J, chromatography, 296, (1984) 369

25. Miwa, M., Tawaki, M., Matsushima, T. and Sugimurua, T. J biol. chem. 249, (1974) 3475

26. Kawaichi, M., Ueda, K. and Hayaishi, O. J. biol. chem. 256 (1981) 9483

27. Purnel, M.R. and Wish, W. J. D. Biochem. j. 185 (1980) 775

28, Liersch, M., Groteluschen, H. and Decker, K. Hoppe-Seyler's z. physiol. chem. 352, (1971) 267

29. Bischoff, E., Tran-Thi, Ti-A. and Decker, K. Eur. j. biochem. 51 (1975) 353

30. Paine, A.J., Allen, C.M., Durcacz, R. W. and Shall, S. Biochem. j. 202 1982) 551

31. Minaga, T., Romaschin, A. D, Kirsten, E. and Kun, E. J. biol. chem. 254 (1979) 9663

32. Romaschin, A.D., Kirsten, E., Jackowski, G. and Kun, E. J. biol. chem. 256 (1981) 7800

33. Wielckens, K., Bredehorst, R., Adamietz, P. and Hilz, H. Eur. j. biochem. 117 (1981) 69

34. Romaschin, A. D. and Kun, E. Biochem. Biophys. Res. Commun. 102 (1981) 952

35. Shall, S. in "DNA repair and its Inhibition" chpt. 9. (1984) 143 (Nucleic Acid Symp. Ser. 13, Eds. Collins, A., Downess, C.S. and Johnson, R.T., IRL Press Oxford, Washington)

36. Butt, T.R. and Smulson, M., Biochemistry 19, (1980) 5235

37. Wong, M., Allan, J. and Smulson, M. J biol. chem. 259 (1984) 7963

38. Adamietz, P. and Rudolph, A. J. biol. chem. 259 (1984) 6841

39. Tanigawa, Y., Tsuchiya, M., Imai, Y., and Shimoyama, M. J. biol. chem. 259 (1984) 2022

40. Kun, E., Chang, A.C. Y., Sharma, M.L., Ferro, A.I. and Nitecki, D. Proc. nat. acad. sci. USA 73 (1976) 3131

41. Niedergang, C., Okazaki, H. and Mandel, P. Eur. j. biochem. 102 (1979) 43

TABLE I

Rates of nicotinamide-release and synthesis of protein adducts of poly(ADP-ribose) by various cell types.

[p mol/ µg DNA]at one minute*

No.	Cell Types	Nicotinamide released	Nicotinamide released in presence of 10mM benzamide	protein bound ADP-ribose
1.	Drosophila K_c	2.2± 0.2	0.0	2.2 ± 0.2
2.	gliosarcoma 9L	40.0± 7.5	7.5± 0.8	12.5 ± 2.5
3.	human fibroblasts	5.0± 0.8	2.0± 0.3	1.8 ± 0.2
4.	mouse spleen lymphocytes (B)	62.0± 12.0	42.0± 9.0	3.0 ± 0.5
5.	rat hepatocytes (6 hr cultures)	150.0± 30.0	139.0± 18.0	2.6 ± 0.4

* n = 5

TABLE II

The balance of NAD and its catabolic products formed by liver nuclei in 10 minutes.

Nucleotide	Retention time in minutes	% total of radio-active material
ADP-ribose[1]	7.6	20.8
AMP[1]	12.9	5.0
NAD[1]	24.4	60.0
Adenosine[1]	32.8	2.7
poly(ADP-ribose)[2]	----	9.0

Recovery = 95.5%. Results are an average of 3 analyses (± 10% SD).

[1] = determined by HPLC (24)

[2] = determined as acid precipitable polymer-protein adducts

Fig 1

73.

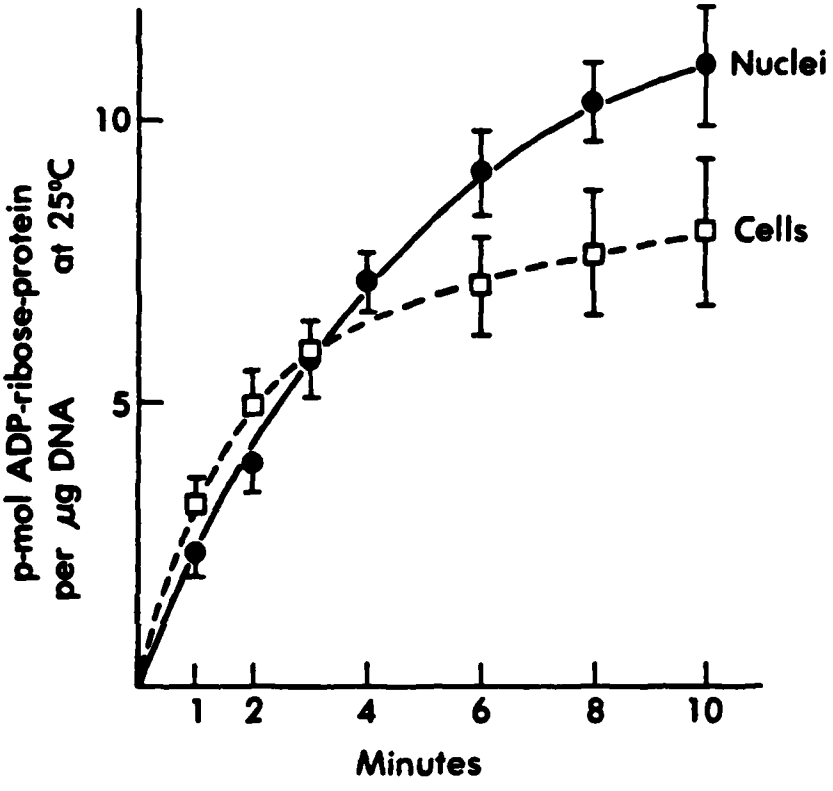

p-mol ADP-ribose-protein per µg DNA at 25°C
10
5
Nuclei
Cells
1
2
4
6
8
10
Minutes

Fig. 2

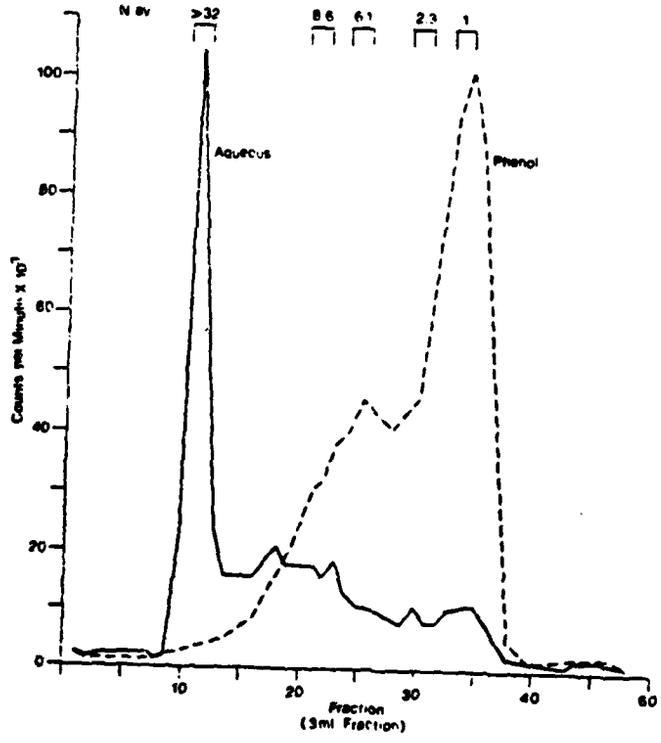
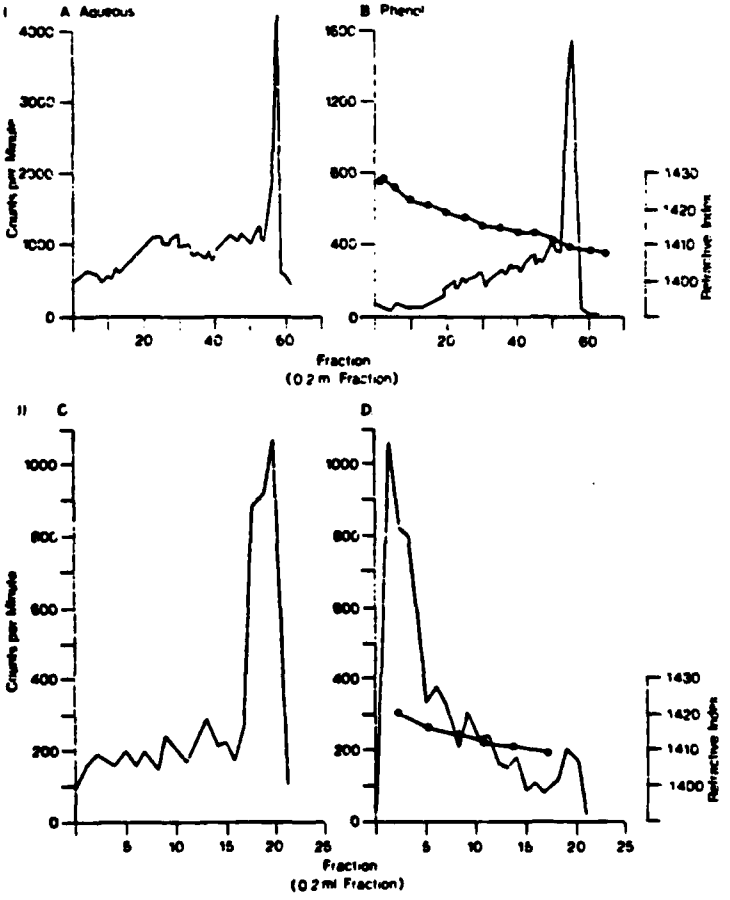
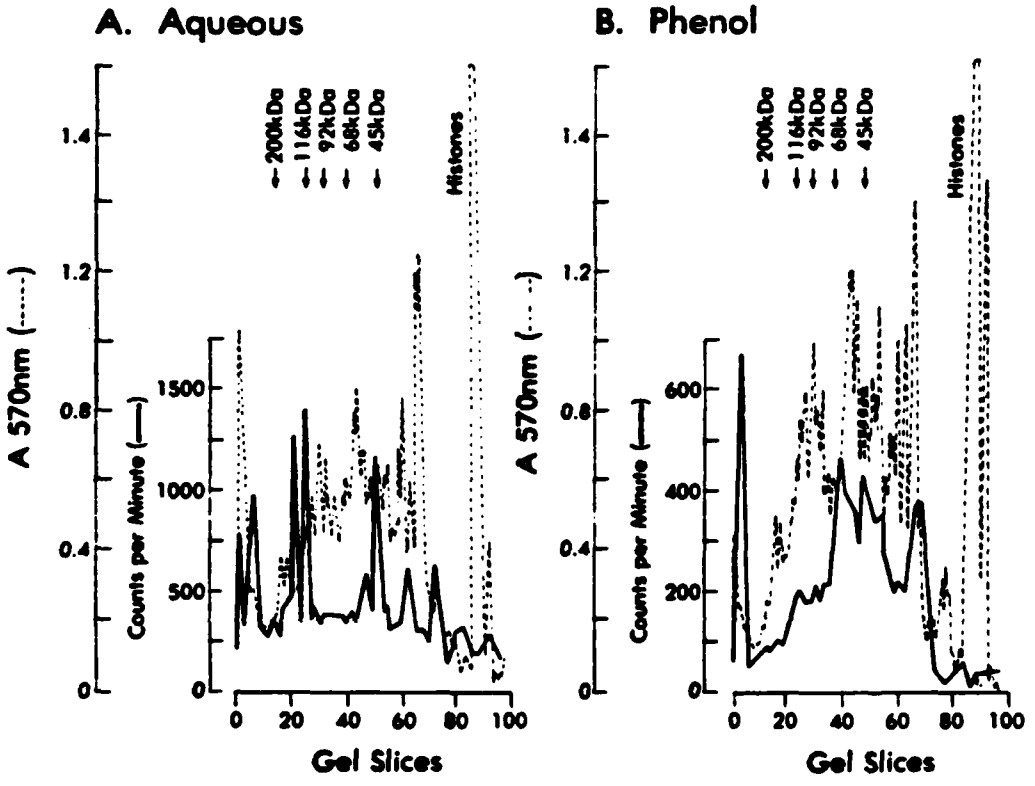
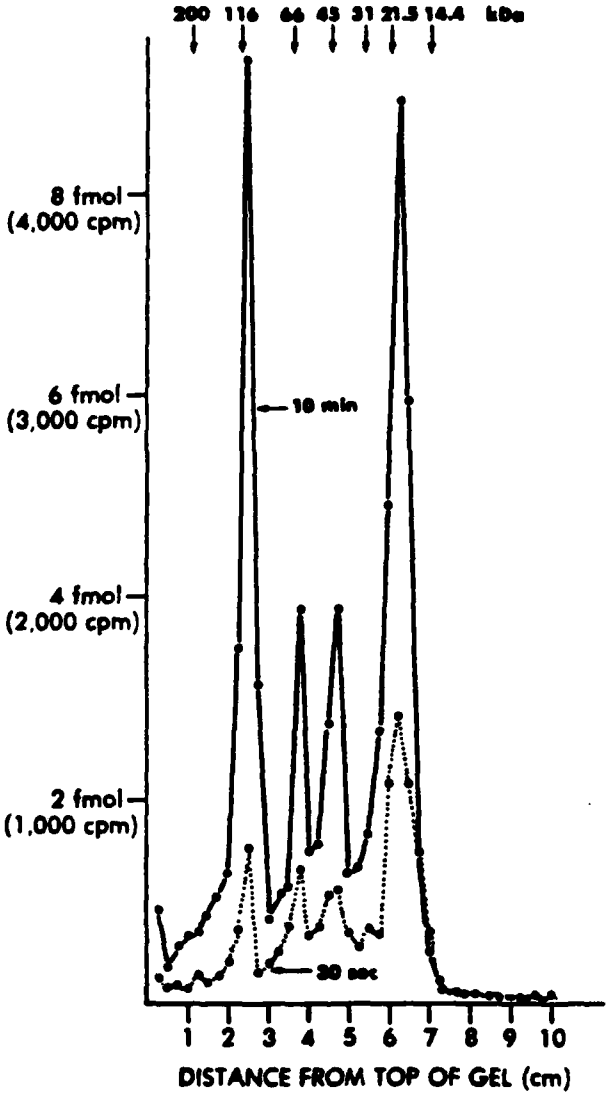

N av
>32
8.6
6.1
2.3
1
100
80
60
40
20
0
Aqueous
Phenol
10
20
30
40
60
Fraction
(3ml Fraction)

Fig. 3


I
A Aqueous
B Phenol
Counts per Minute
4000
3000
2000
1000
0
1600
1200
800
400
0
20
40
60
1430
1420
1410
1400
Refractive Index
Fraction
(0.2 m Fraction)
II
C
D
Counts per Minute
1000
800
600
400
200
0
5
10
15
20
25
1430
1420
1410
1400
Refractive Index
Fraction
(0.2 ml Fraction)

F. 4. 76.


A. Aqueous
B. Phenol
A 570nm (----)
Counts per Minute (—)
200kDa
116kDa
92kDa
68kDa
45kDa
Histones
Gel Slices
0
20
40
60
80
100
0.4
0.8
1.2
1.4
500
1000
1500
200
400
600


200 116 66 45 31 21.5 14.4 kDa
8 fmol
(4,000 cpm)
6 fmol
(3,000 cpm)
4 fmol
(2,000 cpm)
2 fmol
(1,000 cpm)
10 min
30 sec
1 2 3 4 5 6 7 8 9 10
DISTANCE FROM TOP OF GEL (cm)

III.

Molecular studies on the purified poly(ADP-ribose) polymerase system.

1. DNA-association of benzamide .

Abstract.

The interaction of benzamide with the isolated components of calf thymus poly(ADP-ribose) polymerase (3) and with liver nuclei has been investigated. Benzamide-agarose affinity gel matrix was prepared by coupling o-aminobenzoic acid with Affigel 10, followed by amidation. This benzamide-agarose matrix bound the coenzymic DNA but not the purified poly(ADP-ribose) polymerase protein. The binding of benzamide to calf thymus DNA was demonstrated by equilibrium dialysis. Benzoic acid, which is biologically inert does not bind to DNA. Binding of ^{125}I-labeled enzymatically active poly(ADP-ribose) polymerase enzyme protein to DNA-cellulose (8) matrix was not influenced by pretreatment of the DNA-cellulose matrix with various concentrations of benzamide, indicating that the DNA poly(ADP-ribose) polymerase association is not modified by the binding of benzamide to DNA. The binding of benzamide to DNA in liver nuclei was demonstrated. A highly radioactive derivative of benzamide, the ^{125}I-labeled adduct of o-aminobenzamide and the Bolton-Hunter reagent was prepared and its binding to liver nuclear DNA, calf thymus DNA, and specific coenzymic DNA of poly(ADP-ribose) polymerase were compared. Binding to the coenzymic DNA of the ^{125}I-containing ligand was four times higher than to thymus DNA, indicating a degree of specificity of benzamide towards the coenzymic DNA of poly(ADP-ribose) polymerase.

Transformation of synchronized human fibroblasts induced by ultimate carcinogens is prevented by benzamide, when both carcinogens and benzamide are present at non toxic concentrations in the early S phase (10). Although benzamide is known to be a competitive inhibitor of poly(ADP-ribose) polymerase (2) with respect to NAD, its antitransforming effect, that corresponds to an 8-10 μM intracellular concentration (1), appears not to be directly related to an inhibitory action on this enzyme since the poly(ADP-ribose) polymerase activity of cells during the process of prevention of transformation is not inhibited rather increased (1). Induction of poly(ADP-ribose) polymerase in rat liver by benzamide feeding (10) also demonstrates a biological effect of this agent that is unlikely to be related to enzyme inhibition.

A possible cellular mechanism of action of benzamide as an anti-transformer was provided by preliminary experiments with autoradiography. We observed that ^{14}C-labeled benzamide was localized at nuclear membrane sites (1), predicting a probable binding of benzamide to nuclear macromolecules. The present work is concerned with the identification of DNA as the benzamide binding nuclear macromolecule. We also demonstrate that the coenzymic DNA of poly(ADP-ribose) polymerase is a preferred species for benzamide association. The rate and order of magnitude of benzamide-DNA association <u>in vitro</u> approximates conditions found in human fibroblasts during the process of prevention of phenotypic transformation (1) thus the binding of this drug to DNA may have biological relevance.

Experimental Procedures[1]

Results and Discussion

The binding of benzamide to components of the purified poly(ADP-ribose) polymerase was determined with the aid of a benzamide affinity

matrix (see Experimental Procedures). When 0.5 ml of benzamide agarose gel was incubated with either 1.05 mg of poly(ADP-ribose) polymerase of purification step I or with 0.1 mg enzyme protein from purification step IV for 30 minutes at 4°C in the presence of 100 mM Tris-HCl (pH 8.0) 10 mM $MgCl_2$ and 2 mM dithiothreitol (reaction buffer), 94 to 103% enzymatically active protein was recovered in the gel supernatant after centrifugal separation of the gel from eluent, the latter representing non-adsorbed enzyme protein. These results were indistinguishable from the non specific adsorption of traces of the enzymatic protein to agarose, containing no benzamide affinity label. Therefore it was concluded that the enzyme protein does not bind to the benzamide-agarose affinity matrix.

On the other hand, the binding of the specific coenzymic DNA of poly-(ADP-ribose) polymerase to the benzamide-agarose was readily demonstrable. The binding of DNA to the affinity column was determined by an enzymatic test which quantitatively assays the unbound coenzymic DNA in a reconstructed in vitro system, containing purified polymerase protein as a catalyst. A constant amount (0.2 or 0.4 ml) of benzamide-agarose was incubated with a 2.3 µg coenzymic DNA in a total volume of 0.64 ml reaction mixture composed of Tris-Mg^{2+}-dithiothreitol reaction buffer (see above) for 30 min. at 25° with sustained agitation. At the end of this period, the affinity gel was spun down at 2,000 x g for 10 min. and 200 µl of the supernatant tested for coenzymic DNA content in a test system containing 32.5 µg polymerase (stage IV) protein/ml, 108 µM NAD (^{14}C-labeled in the adenine moiety, 2.5 µCi per ml) at 25° in a reaction volume of 400 µl. Aliquots of 70 µl were assayed for the quantity of auto-poly ADP-ribosylated enzyme protein as shown in Fig. 1. The rate of auto-poly ADP-ribosylation of the enzyme was

igure 1 here

linear with time and was a linear function of the concentration of free coenzymic DNA in this system below saturation of the enzyme with DNA. The top linear curve shows results of the control experiment, where DNA was incubated with 0.4 ml of agarose gel containing no benzamide affinity label[2], whereas the second and third curves indicate diminishing DNA content of the gel supernatant, demonstrating the binding of DNA to increasing quantities of the benzamide gel matrix. The experiment shown in the fourth curve contains no added DNA in the incubation system, only the gel supernatant. In separate experiments it was demonstrated that incubation of the benzamide affinity gel with the buffer does not release an enzyme inhibitor, possibly o-aminobenzamide, since 100% enzymatic activity could be recovered when gel supernatants were added to the standard poly(ADP-ribose) polymerase catalytic system (see insert of Fig.1). The DNA that was bound to the affinity column could be quantitatively recovered from the gel by overnight incubation with 1 M NaCl at 4°. Furthermore, traces of commercial calf thymus DNA could be bound to the affinity gel and after recovery with 1 M NaCl the eluted DNA exhibited coenzymatic function in the poly(ADP-ribose) polymerase test. It should be noted that the enzymatic test for the binding of coenzymatically active DNA to the benzamide-agarose matrix by necesssity indicates a certain degree of specificity. The enzymatic assay for the specific coenzymic DNA could not have detected an amount of non-specific, e.g. unfractionated calf thymus DNA at concentrations consistently used for the benzamide gel binding experiments of coenzymic DNA.

In the next series of experiments we tested the possibility of interference by benzamide with the DNA-association of the purified enzyme protein. This test was carried out with ^{125}I-labeled enzyme (stage V) and DNA-

cellulose affinity matrix(8). First the association of ^{125}I-labeled enzymatically 95% active poly(ADP-ribose) polymerase protein to DNA-cellulose was determined. Varying concentrations (from 0 to 78 nM) of enzyme protein (107 cpm ^{125}I/f mol of enzyme) were incubated with a constant amount of DNA-cellulose in 270 µl total volume of reaction mixture containing 7 µg DNA/ml resin suspension, 100 mM Tris-HCl, 10 mM $MgCl_2$, 2 mM dithiothreitol and 100 µg/ml bovine serum albumin for 20 min. at 25°C. For displacement (i.e. determination of specific binding), control tubes contained in addition to the above components 250 µg/ml calf thymus DNA. At the end of incubation the resin was spun down for 4 min. at 2500 x g and the bound and free enzyme were determined by ^{125}I counts. Results are given as a Scatchard plot in Fig.2 and K_D was calculated to be 3.7 nM.

Figure 2 here

In subsequent experiments the binding of 3.6, 14.4 and 33.2 nM ^{125}I-labeled poly(ADP-ribose) polymerase protein to DNA-cellulose were determined following incubation of DNA-cellulose with 0 to 1 mM benzamide for 20 hours. Results (Fig.3) clearly indicate that benzamide did not interfere with the specific association of the polymerase protein with DNA.

Figure 3 here

The association of benzamide with calf thymus DNA was also determined by equilibrium dialysis. Calf thymus DNA (1 mg/ml) was dissolved and extensively dialyzed against the reaction buffer: 150 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$ and 2 mM dithiothreitol. Aliquots of 200 µl of this DNA solution were pipetted into dialysis bags (Spectrapor, mol. wt. cut off at 12,000) and these were placed into 5 ml aliquots of the above buffer, containing varying concentrations of ^{14}C-labeled benzamide (0-110 µM). Quenching of the specific binding of ^{14}C-labeled benzamide to DNA was determined by the addition of 5 mM non-radioactive benzamide. The dialysis

lasted 40 hours at 4°. Both DNA content and radioactivites were determined in each dialysate and specific binding expressed in a Scatchard plot as illustrated in Fig.4. The insert demonstrates the effect of varying concentration of added benzamide on its binding to calf thymus DNA. A calculated binding constant of 547 μM was obtained from the Scatchard plot (Fig.4). This value, if converted to ½ saturating concentration of benzamide per weight of DNA is 2.7 μM/μg DNA, that approximates cellular conditions where benzamide acts as an antitransformer (1). This type of calculation by necessity yields only average values, because it does not take into account much more efficient benzamide binding to specific DNA domains, a condition indicated by the four-fold increase in benzamide binding to the coenzymic DNA species, as compared to unpurified thymus DNA (see later). The process of binding was slow with estimated $t_{\frac{1}{2}}$ of 10 hours. When this experiment was repeated with ^{14}C-labeled benzoic acid, instead of benzamide, no association of benzoic acid to DNA was detectable. Benzoic acid was not an antitransformer in cell culture experiments either (1).

Figure 4 here

The DNA concentration dependence and time course of binding of benzamide to DNA, present in isolated liver nuclei, is illustrated in Fig.5. The time of incubation was 2.5 h at 25° in order to minimize DNA breakdown. Increasing quantities of nuclei proportionally to their DNA content bind more benzamide (Fig. 5). The rate of binding was linear with time up to 8 hours (insert of Fig.5) a time limit chosen because significant decay of nuclear DNA is likely beyond this period.

Figure 5 here

The higly radioactive ^{125}I-labeled Bolton-Hunter reagent derivative of o-aminobenzamide (see Experimental Procedures) was tested as a ligand for DNA present in liver nuclei and its association with coenzymic DNA and with commercial calf thymus DNA were also compared. When increasing

quantities of liver nuclei, equivalent to 66,99 and 132 µg DNA were incubated for 8 hours with 0.96 mM non radioactive iodinated Bolton-Hunter reagent adduct of o-aminobenzamide, charged with tracer quantity of ^{125}I containing adduct (603 MBq/µg) equivalent to 3.8×10^6 cpm in 100 µl Tris-Mg-dithiothreitol buffer (see above), 0.33, 0.68 and 1.1 n mol ligand was proportionally bound to DNA as a function of the quantity of DNA in liver nuclei. In these experimentas 10 mM unlabeled non radioactive iodinated ligand was used for quenching. It is apparent that the ^{125}I containing ligand, which has a specific radioactivity 10 times that of ^{14}C-labeled benzamide, is suitable for DNA binding studies. It was of obvious importance to ascertain that DNA in liver nuclei is the only macromolecular species that binds benzamide. To test this we exposed liver nuclei briefly to DNA-ase I and determined the rate of DNA degradation by direct chemical analysis (7). Simultaneously we also assayed the binding of the ^{125}I-labeled ligand to untreated nuclei and to nuclei which were exposed to DNA-ase I. Results of two separate experiments, which gave identical values, are shown in Table II. Reducing DNA content of nuclei to about 1/5 completely abolished the binding of the ^{125}I-labeled benzamide derivative to liver nuclei. No detectable proteolysis occurs under the same conditions.

If the association of benzamide to DNA has relevance to poly ADP-ribosylation related control mechanisms in cellular systems, it would be expected that the coenzymic DNA of poly(ADP-ribose) polymerase should exhibit some degree of specificity of benzamide binding. We have tested this possibility by comparing the association of the ^{125}I-labeled ligand with calf thymus DNA and with purified coenzymic DNA. Because of the

limited availability of specific coenzymic DNA we carried out equilibrium dialysis only at one concentration of DNA (1 mg/ml) and ligand. The experiment was similar to that described for Fig.4, except the volume of dialysis bags was 200 µl and the volume of dialysis buffer 5 ml, containing 9.6×10^6 cpm ^{125}I-labeled ligand at a final concentration of 0.1 mM. The time of dialysis was 44 hours at 4°C. Coenzymic DNA bound 60 p mol ^{125}I-ligand per 100 µg DNA whereas unpurified calf thymus DNA bound only 16 p mol/ 100 µg DNA. These results confirm the predicted increased binding of benzamide to the coenzymic DNA species. The identification of probable structural components of DNA required for benzamide binding is subject of further studies.

In preliminary experiments* with proton NMR analysis in $CDCl_3$ it was shown that benzamide interacts with 9-ethyladenine identically to the base pairing between 9-ethyladenine and 1-methyl thymine, therefore independent physical evidence supports the association of benzamide with DNA bases as determined by binding equilibria and kinetics.

Biological effectivity of certain antibiotics is known to correlate with their binding to selective DNA sequences (9). Therefore the pharmacological action of a non toxic concentration of benzamide, in preventing probably initiating events that lead to cellular transformation, could be correlated to a binding of benzamide to specific DNA sites, a problem subject to further experiments. Benzamide (k_i = 39 µM), o-amino-benzamide (k_i = 71 µM) and the adduct of o-aminobenzamide with the Bolton-Hunter reagents (k_i = 152 µM) are competitive inhibitors of poly(ADP-ribose) polymerase with respect to NAD. It is unlikely that the binding of benzamide

*Unpublished experiments by J. McLick and E. Kun

to DNA has any relationship to the enzyme inhibitory effects of these substances, determined by initial velocity kinetics. Enzyme inhibition was determined within 1-2 minutes whereas $t_{\frac{1}{2}}$ of benzamide binding to DNA is about 10 h, a time constant which coincides with the temporal requirement of prevention of cellular transformation (1).[3]

REFERENCES

1. Kun , E., Kirsten,E., Milo,G.E., Kurian,P., and Kumari,H.L. (1983) Proc. Natl. Acad. Sci. USA 80, 7219-7223.
2. Purnell,M.R. and Wish,W.J.D. (1980) Biochem. J. 185, 757-777.
3. Yoshihara,K., Hashida,T., Tanaka,Y., Ohgushi,H., Yoshihara,H. and Kamiya,T. (1978) J. Biol. Chem. 253 6549-6466.
4. Liew,C.C. and Chan,P. (1976) Proc. Natl. Acad. Sci. USA 76, 3458-3462.
5. Hakam,A., McLick,J. and Kun,E. (1984) J. Chromatography 296, 369-377.
6. Bradford,M. (1976) Anal. Biochem. 72, 248-254.
7. Labarca,C., Paigen,K. (1980) Anal. Biochem. 102, 344-352.
8. Alberts,B. and Herrick,G. (1971) Methods in Enzymology XXI (Eds. Grossman ,M.J. and Moldave,K.) Academic Press, NY, pp.198-217.
9. Waring,M.J. and Fox,K.R. (1983) in Topics in Molecular and Stuctural Biology. (Ed. Neidle,S.) MacMillan Publ., London , Vol.3, pp.127-156.
10. Griffin,M.J., Kirsten,E., Carubelli,R., Palakodety,R.B., McLick,J. and Kun,E. (1984) Biochem. Biophys. Res. Commun. (in press).

EXPERIMENTAL PROCEDURES: THE ASSOCIATION OF BENZAMIDE WITH DNA.

BAUER,P.I., HAKAM,A. AND KUN,E.

1. Calf thymus poly(ADP-ribose) polymerase and enzyme associated active DNA were isolated according to Yoshihara et.al. (3). The procedure and its results are illustrated in Table I.

Purification Stages	Preparation	Volume ml	Protein mg	Specific Activity*	Total Activity*	Purification	Yield
I	crude extract	800	32540.0	1.3	42300	1.0	100%
II	$(NH_4)_2SO_4$	300	9150.0	4.2	38430	3.2	91%
III	DNA-cellulose	350	129.5	224.0	29008	172.0	68.5%
IV	OH-apatite	4	8.5	1120.0	9520	861.0	22.5%
V	Sephacryl-S 200	3	4.5	1500.0	6750	1153.0	15.9%

*n mol ADP-R/mg protein x min.

The enzyme at stage V is 95% homogenous, as determined by gel electrophoresis, with no contaminants at the 25 kDa mass region.

Active DNA was obtained by eluting the OH-apatite column after the enzyme elution with a linear phosphate gradient (30→300 mM) in a buffer composed of 50 mM Tris-HCl pH 7.4, 1 mM EDTA, 1 mM Na-azide, 1 mM reduced glutathione, 0.5 mM dithiothreitol, 2 M KCl. Fractions containing the DNA were dialyzed against 10 mM Tris-HCl pH 7.4, 1 mM EDTA buffer, then freeze dried. Yield: 3.65 mg DNA, which is estimated to be 0.9 n mol, with an estimated DNA size of 3-4 kb (double stranded), (unpublished results).

2. Rat liver nuclei were isolated according to Liew and Chan (4) as modified by Hakam et.al. (5). The protein content (6) of the nuclear suspensions used routinely was 29 mg/ml, the DNA content 10 mg/ml, the latter determined by fluorometry (7). The poly(ADP-ribose) polymerase activity was within ± 20% of 1.86 n mol ADP-ribose bound per mg protein per min. assayed in a test system composed of 150 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 1.5 mM dithiothreitol, and varying quantities of ^3H or ^{14}C (in the adenine moiety) labeled NAD as specified in the legends. The enzyme activity was determined at 20°, terminated with 1 ml 10% trichloroacetic acid (4°) and precipitable radioactive material assayed either directly, or after separation of poly(ADP-ribose) by high performance liquid chromatography (5). When the purified enzyme was tested the assay system also contained 10 µg/ml specific DNA as a coenzyme.

3. Synthesis of o-aminobenzamide-agarose affinity matrix. Anthranilic acid (2.56 g) was dissolved in 50 ml of 0.5 M $NaHCO_3$ and the pH adjusted to 10.7 with 2 N NaOH. Affigel 10 (Bio Rad Labs) was suspended and washed in the $NaHCO_3$-NaOH buffer then 20 ml of the gel suspension (settled by gentle suction filtration) was added to the

anthranilic acid solution and the mixture slowly agitated for 3 hours at room temperature, then left overnight at 4°C. The gel was washed successively with 200 ml portions of 1 M Na_2HPO_4, H_2O, 1 M KH_2PO_4, H_2O and finally with 1 M $(NH_4)_2SO_4$. 20 ml of the wet gel was suspended in 40 ml of 1 M $(NH_4)_2SO_4$ and EDAC [1-Ethyl-3-(3-dimethylamino-propyl)-carbodiimidel] was added to a final concentration of 0.1 M , the pH adjusted to 4.7, thereafter maintained between 3.3 and 5.5. during gentle agitation at room temperature for 4 hours, and the suspension left overnight at 4°C. The gel was again washed with 200 ml portions of 1 M Na_2HPO_4, H_2O, KH_2OO_4 and H_2O. The quantity of agarose bound o-aminobenzamide was determined by two procedures. (A), aliquots of the gel were suspended in glycerol and its absorption spectrum determined. The quantity of gel/cuvette was adjusted on the basis of equal light scattering at A_{450nm}. The concentration of the ligand was calculated to be 9.2 µ mol per ml Affigel 10. (B), the o-aminobenzamide gel was hydrolyzed in 6 M HCl at 100°C for 48 hours, thereafter the sample was freeze-dried, dissolved in H_2O and again freeze dried. This process was repeated 3 times, and finally the material was taken up in 1 ml H_2O, pH was adjusted to 7.5-8.0. An aliquot was analyzed by high performance liquid chromatography (ALtex, Ultrasphere-ODS column, 4.6 mm x 25 cm) and resolved with a linear gradient of methanol (0-25%) in 50 mM phosphate, pH 4.25, time = 40 min. One ml fractions were collected and analyzed fluorometrically (374 nm excitation, 420 nm emission) in 25% glycerol, and compared to anthranilate standards, prepared by adding appropriate quantities of anthranilate plus Affigel 10, which was treated identically with the samples to be analyzed. The amount of o-aminobenzamide bound per ml gel was calculated to be 7.3 µ mols per ml in reasonable agreement with results obtained by (A).

4. DNA-cellulose was prepared according to Alberts (8) and the quantity of cellulose bound DNA, determined spectrophotometrically (following hydrolysis for 4 hours at 90° in 10 mM phosphate + 1 mM EDTA at pH 7.4) was 0.7 mg DNA per ml resin.

5. Iodination of poly(ADP-ribose) polymerase protein. 10 to 20 µg of IODO-GEN (Pierce, 1,3,4,6-tetrachloro-3α,6α-diphenylglyco-uril) was dissolved in 200 µl $CHCl_3$ and the solvent evaporated, leaving a film on the surface of the tube. 200 µg purified enzyme protein dissolved in 133 µl 100 mM Tris-HCl (pH 7.4) 150 mM NaCl was added and incubated for 10 min. at 4°C with 1 mCi ^{125}I-NaI, the latter added in a volume of 10 µl. The enzyme protein was separated from the unreacted ^{125}I by gel filtration on a Sephadex G-75 column (0.7 x 6 cm), equilibrated with the Tris-NaCl buffer, supplemented with 2 mM dithiothreitol. Enzymatic activity of the iodinated enzyme was determined by chromatographic analysis of the polymer following NaOH and proteinase K hydrolysis of the polymer-protein adducts (cf.5). After iodination more than 95% activity was recovered.

6. Preparation of the Bolton-Hunter (BH) reagent [3(p-hydroxyphenyl)propionyl-N-hydroxy succinimide ester] adduct of o-aminobenzamide and its iodination.

a. Synthesis. 1 g of BH reagent (38 m mol) was reacted with 2.72 g (20 m mol) anthranilamide in 150 ml of absolute ethanol by leaving the reaction mixture overnight at room temperature. The product was isolated on preparative TLC silica gel plates (Analtech) with a developing solvent composed of $CHCl_3$, MeOH, CH_3COOH (90;10:2). The separated product was scraped off the plate and extracted with ethanol for 2 hours at room temperature and the solvent was evaporated from the pooled eluates. Final purification is achieved by rechromatography which removes possible traces of unreacted anthranilamide. The product gave a single spot on TLC using the above developing solvent. Rf for anthranilamide = 0.61; 0.45 for BH-adduct of anthranilamide; the BH reagent decomposes to two products Rf: 0.31, 0.87.

b. Iodination. 50 µg of IODO-GEN was dissolved in 100 µl $CHCl_3$, and the solvent evaporated in order to coat the inner surface of a tube with the reagent. Twenty-five µg (88 n mol) BH-anthranilamide was dissolved in 25 µl 1 M NaAc (pH 5.6) and added to the iodogen coated tubes followed by 1 mCi ^{125}I-NaI. The reaction was allowed to proceed for 10 min. at room temperature then products were separated by TLC in the system described above. The radioactive product was located by autoradiography (Rf 0.85) and eluted with ETOH; assuming the same specific activity of ^{125}I in the product as in ^{125}I-NaI, its quantity was 0.195 n mol (43% yield).

7. The Benzamide-Ring UL ^{14}C-labeled product and benzoic acid (7.2 mCi/m mol) were purchased from Pathfinder Labs 11542 Fort Mims Drive, St. Louis, MO. All other reagents were of analytical grade. ^{14}C-labeled NAD was obtained from Amersham Corp. Arlington Heights, IL; ^{32}P-labeled NAD from New England Nuclear Corp., Boston, MA. ^{125}I was purchased from Amersham Corp., Arlington Heights, IL.

Table II

Correlation between DNA content of liver nuclei prior and after digestion with DNA-ase I with the binding of the ^{125}I labeled adduct of the Bolton Hunter Reagent and o-amino benzamide.

	Time of incubation with DNA-ase I (min)	DNA content (µg DNA/mg protein)	^{125}I-ligand binding (p mol/mg protein)	% decrease in binding
1.	0	345	981	0
2.	10	242	392	60
3.	30	76	0	100

LEGENDS

Figure 1. Binding of specific coenzymic DNA to benzamide-agarose matrix. The binding of DNA to benzamide agarose was assayed enzymatically as described in Results and Discussion. Ordinate: quantity of enzymatic product that is the auto-poly ADP-ribosylated enzyme protein; abscissa: time of enzymatic reaction at 25°. -●-● : eluate of agarose, containing no ligand (control). -○-○ : eluate of 0.2 ml benzamide agarose incubate. -■-■ : eluate of 0.4 ml of benzamide agarose incubate. -□-□ : eluate of benzamide agarose in the absence of added DNA (control 2). Insert indicates that no benzamide was released from benzamide-agarose matrix during incubation. In this experiment benzamide-agarose or agarose were incubated without added DNA (0 to 10 min. = abscissa) in the same system as described in Results and Discussion. Aliquots of the supernatant were tested in the poly(ADP-ribose) polymerase assay in order to determine possible "leakage" of enzyme inhibitor from the benzamide gel. Ordinate: % enzymatic activity in the absence or presence of gel eluates. The enzyme assay contained 10 μg/ml specific coenzymic DNA.

Figure 2. Binding of ^{125}I-poly(ADP-ribose) polymerase to DNA-cellulose. Details of the experiments are described in Results and Discussion, the figure represents a Scatchard analysis.

Figure 3. The absence of an effect of benzamide on the binding of ^{125}I-poly(ADP-ribose) to DNA-cellulose. DNA cellulose was preincubated in the reaction buffer with varying concentrations of benzamide for 20 hours at 4° prior to the enzyme binding test, as done in experiments described above (Fig.2). -●-● : 33.2 nM enzyme. -○-○ : 14.4 nM enzyme. -■-■ : 3.6 nM enzyme.

Figure 4. The binding of ^{14}C-benzamide to calf thymus DNA, determined by equilibrium dialysis. For details seee Results and Discussion. Insert shows the dependence of binding on the concentration of benzamide.

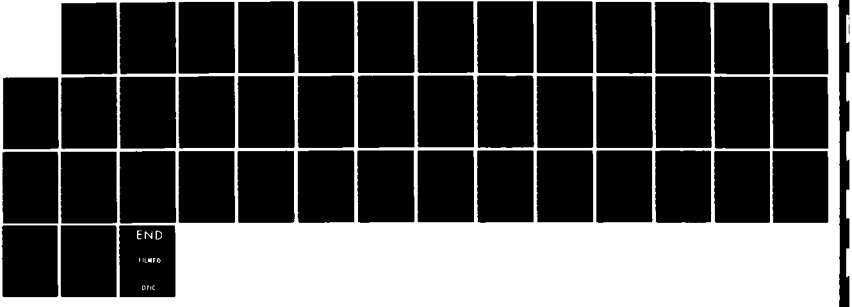
Figure 5. The binding of ^{14}C-benzamide to DNA of rat liver nuclei. Increasing amounts of liver nuclei containing 0.1 to 0.5 mg DNA (abscissa) were incubated with 3 mM ^{14}C-labeled benzamide at pH 8.0 in the reaction buffer (see Results and Discussion) for 2.5 hours at 25° in the presence or absence of 20 mM unlabeled benzamide as a quenching agent. One ml of cold incubation buffer, containing 1 mg serum albumin per ml was added and nuclei rapidly sedimented (30 sec.) in a Beckman microfuge. The pellet was washed, then dried, the tip of the Eppendorf centrifuge tube cut off and radioactive material determined by scintillation counting following 12 h solubilization in the scintillator. The time curve of benzamide binding nuclei equivalent to 0.2 mg DNA is shown in the insert.

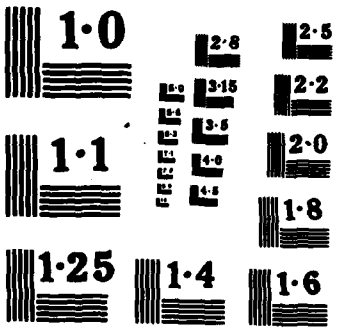
Table II

Liver nuclear suspension (955 µg protein/500 µl) incubated with DNA-ase I (5 µg/ml) in the presence of 5 mM $CaCl_2$ and 10 mM $MgCl_2$, 150 mM Tris-HCl pH 8.0, 2 mM DTT, at 25°C in a total volume of 500 µl. At intervals given in the Table aliquots of 150 µl were withdrawn, mixed with 1 ml of buffer (Tris-Mg-DTT) containing 10 mM EGTA and centrifuged (10 min, 1100 x g , 4°). The pelleted nuclei were resuspended in the buffer containing 1 mM EGTA and their ^{125}I-BH-ligand binding capacity was tested. Experimental conditions were as follows: nuclei (66 µg DNA, 191 µg protein) were incubated in 0.96 mM ^{125}I-ligand (4.0×10^6 cpm) at 25°C for 3.5 hours in a volume of 100 µl. The subsequent techniques were identical as described in the legend of Fig.5.

AD-A154 415 MOLECULAR TOXICOLOGY OF CHROMATIN: THE ROLE OF POLY(ADP-RIBOSE) IN GENE CONTROL(U) CALIFORNIA UNIV SAN FRANCISCO E KUN FEB 85 AFOSR-TR-85-0467 2/2

UNCLASSIFIED F49620-81-C-0007 F/G 6/20 NL




1·0
1·1
1·25
1·4
1·6
1·8
2·0
2·2
2·5
2·8
3·15
3·5
4·0
4·5

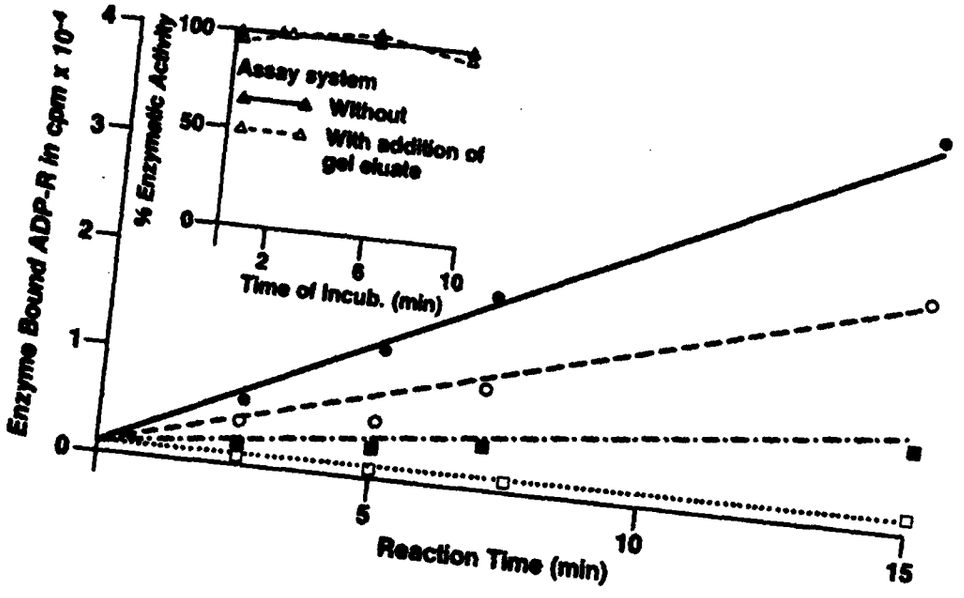

Enzyme Bound ADP-R in cpm x 10⁻⁴
4
3
2
1
0
5
10
15
Reaction Time (min)
% Enzymatic Activity
100
50
0
2
6
10
Time of Incub. (min)
Assay system
Without
With addition of gel eluate

Fig 1.

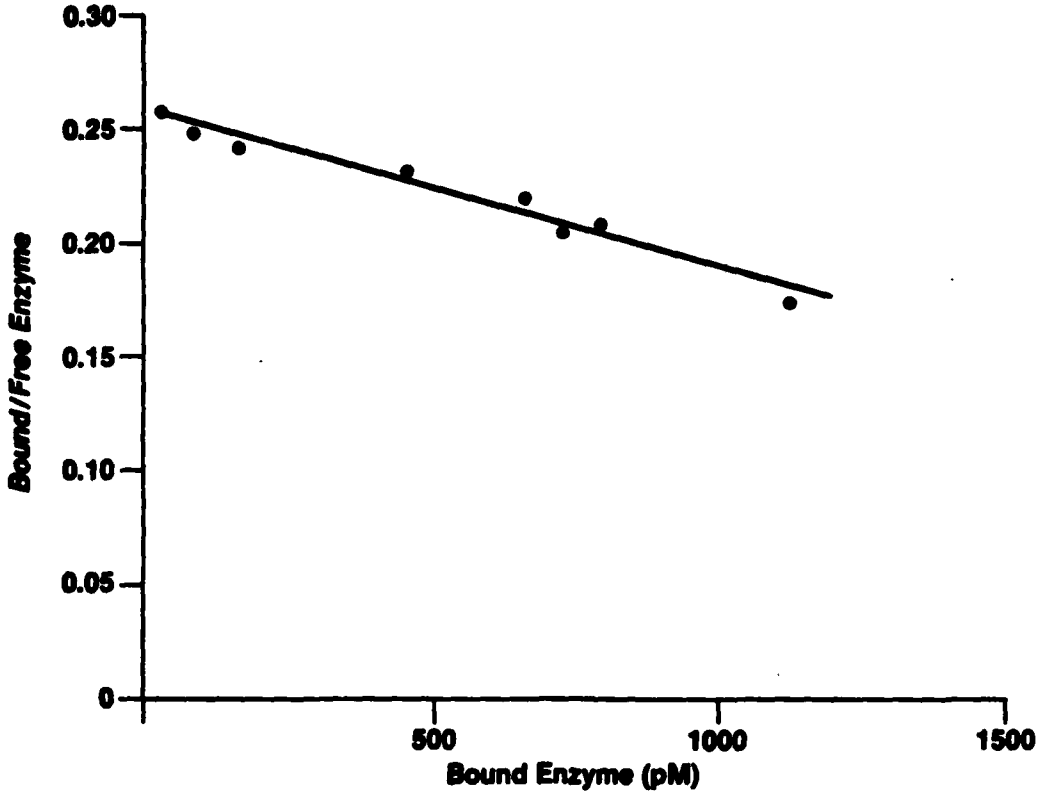

0.30
0.25
0.20
0.15
0.10
0.05
0
Bound/Free Enzyme
500
1000
1500
Bound Enzyme (pM)

Fig 2

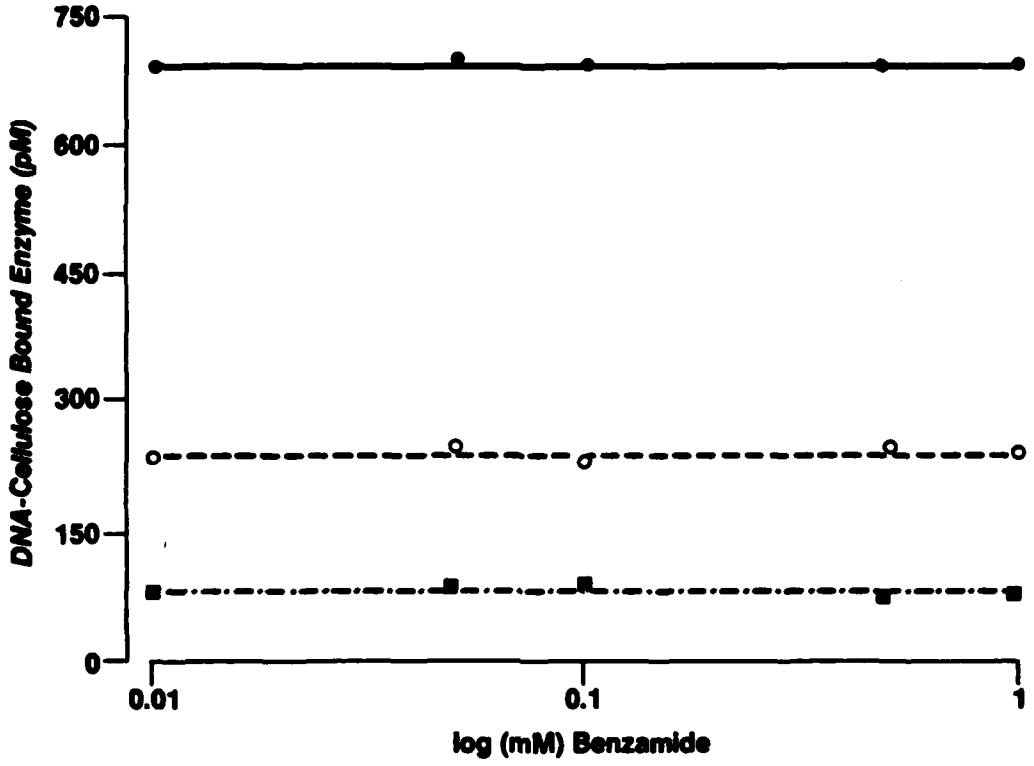

750
600
450
300
150
0
DNA-Cellulose Bound Enzyme (pM)
0.01
0.1
1
log (mM) Benzamide

Fig 3.

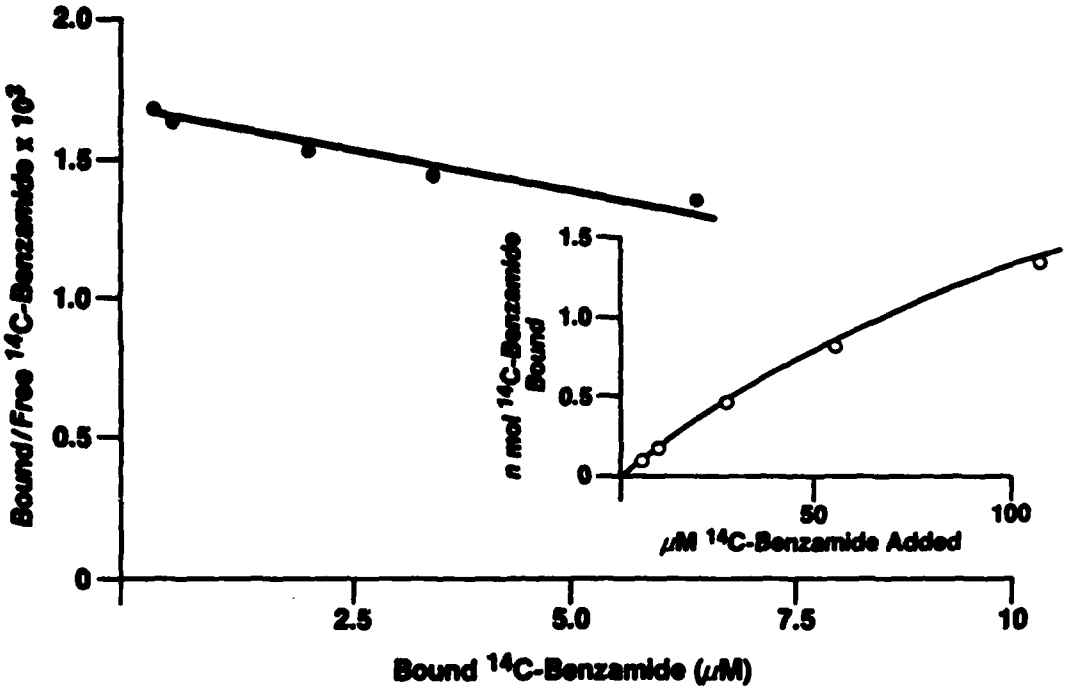

Bound/Free ^{14}C-Benzamide x 10^3
2.0
1.5
1.0
0.5
0
2.5
5.0
7.5
10
Bound ^{14}C-Benzamide (μM)
n mol ^{14}C-Benzamide Bound
1.5
1.0
0.5
0
50
100
μM ^{14}C-Benzamide Added

Fig. 4.

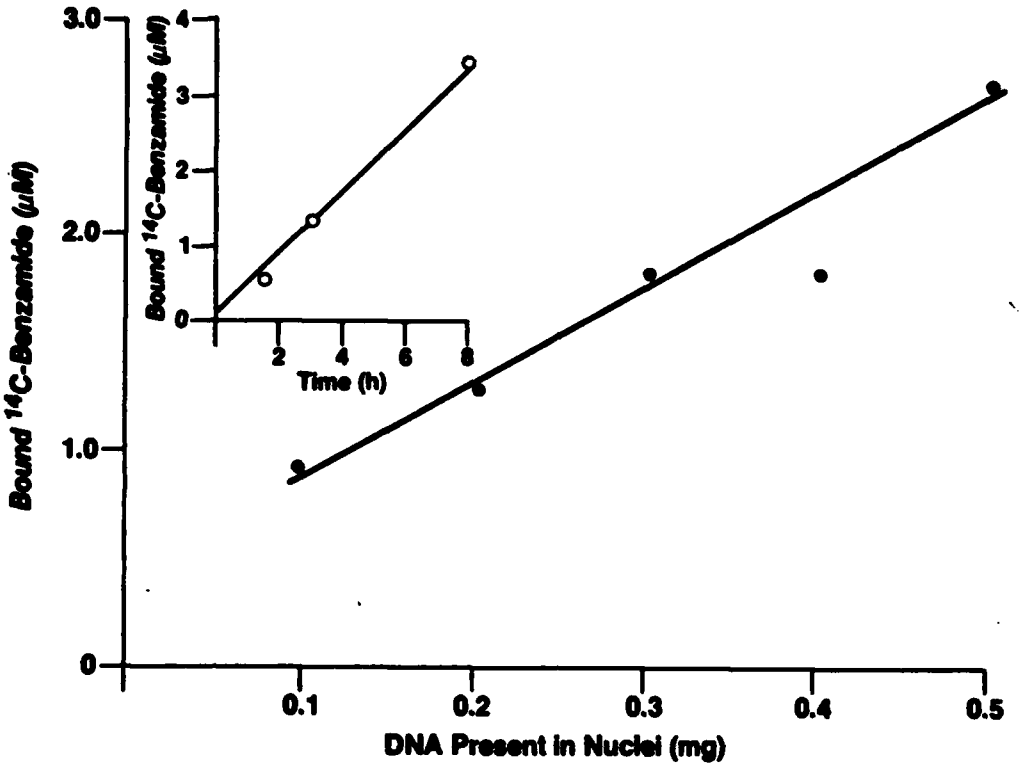

Bound ^{14}C-Benzamide (μM)
3.0
2.0
1.0
0
0.1
0.2
0.3
0.4
0.5
DNA Present in Nuclei (mg)
Bound ^{14}C-Benzamide (μM)
4
3
2
1
0
2
4
6
8
Time (h)

Fig 5.

2. The role of lysine residues in the catalysis and DNA binding of poly(ADP-ribose) polymerase.

Summary

Chemical modification of 94% of the terminal lysine groups of the purified poly(ADP-ribose) polymerase enzyme protein primarily inhibited enzymatic polymerization of ADP-ribose and the binding of coenzymic DNA to the enzyme protein thereby indentifying a specific DNA as an elongation coenzyme. The second catalytic action of the polymerase protein, NAD-glycohydrolase activity, was not significantly dependent on intact lysine end-groups nor on DNA. Methylacetimidated polymerase protein, although inactive as a polymerization catalyst, can still serve as a polymer acceptor substrate for poly (ADP-ribose) in the presence of matrix-bound fully active poly(ADP-ribose) polymerase enzyme, providing a molecular model for protein poly ADP-ribosylation. Two types of ADP-ribose-protein bonds were identified on the basis of susceptibility to hydroxylaminolysis. The ester type bonds are likely to be generated by the initiation step of enzymatic polymerizatiom, whereas the Schiff base type adducts formed from free ADP-ribose addition to lysine residues, mediated by the NAD-glycohydrolase action of the polymerase, constitute the second type (hydroxylaminolysis resistant) elongation templates. A molecular model for the regulation of nuclear poly-ADP-ribosylation of chromatin proteins is proposed on the basis of chemical and enzymatic modification of lysine terminals of the enzyme protein.

The eukaryotic nuclear specific poly(ADP-ribose) polymerase (E.C. 2.4.99) enzyme catalyzes the transfer of ADP-ribose from NAD to various nuclear protein acceptors, including the enzyme protein itself, and continues elongation to homopolymers of variable length (1,2,3). The purified enzyme requires a specific coenzymic DNA for catalytic activity (4). Besides the unique polymerization of ADP-ribose the polymerase enzyme protein catalyzes also the extra or abortive hydrolysis of NAD, and experimental evidence indicates that the two types of catalytic functions are associated with the same enzyme molecule (5). Molecular mechanisms that could explain the duality of the catalytic function of the enzyme as well as the coenzymic role of DNA have not been clarified. The branched (6) and helical conformation (7,8) of the enzymatic product, poly(ADP-ribose), have been established, and indications exist that these structural features may have cellular biological significance (9). However experiments related to molecular mechanisms of the enzymatic process of polymerization are scanty. As a part of research concerned with enzymatic mechanisms we report here that lysine residues of the polymerase protein play a critical function with respect to DNA binding, identifying the coenzymic DNA as a predominantly chain elongating cofactor. The intactness of lysine residues also determines whether mono or poly ADP-ribose adducts are generated by the enzyme protein. NAD-glycohydrolase and polymer accepting properties of the enzyme are retained when polymerization is inhibited by the modification of lysine end groups, indicating a lysine residue dependent interconversion of catalytic properites. It was deduced that the polymerase most probably catalyzes the formation of ester bonds only, whereas the NAD-glycohydrolase activity of the enzyme by producing free ADP-ribose can generate Schiff base type adducts with proteins (10) which are known to serve as elongation templates (11). This duality of templates explains the occurrence of the frequently

observed hydroxylamine sensitive and hydroxylamine stable protein ADP-ribose adducts (cf 3, 15). The specific lysine modifying reagent methyl acetimidate (12) proved to be a useful experimental tool, since it is known that out of 118 amino terminals of the enzyme 117 are lysine residues (13).

Experimental Procedures

Preparative and Enzymatic Methods.

Poly (ADP-ribose) polymerase and coenzymic DNA were purified from calf thymus by the method of Yoshihara et al. (4). The enzyme preparation was at least 95% pure with no contamination by low molecular weight proteins and had an activity of 1066 nmol ADP-ribose incorporated/mg protein/min. For catalytic assays, (7,8,9) the enzyme (0.5 - 2 µg) was diluted with a solution of 100 µg/ml albumin, and incubated with 10 to 50µM NAD (65 µCi/µmol, ^{14}C in the adenine ring), 150 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 0.5 mM DTT and 10 µg/ml coenzymic DNA at 23°C. The NAD glycohydrolase activity was determined by withdrawing at various intervals 5 µl aliquots which were spotted onto PEI-cellulose (Macherey-Nagel, Duren, FRG) TLC sheets and developed either with 0.1 M K phosphate pH 3.4, or with 0.9 M CH_3COOH 0.3 M LiCl buffers. Poly (ADP-ribose) remained at the origin and ADP-ribose (localized by authentic ADP-ribose) was cut out, eluted with 1 ml of 2 M LiCl and counted in Aquasol.

Modification of poly (ADP-ribose) polymerase with methyl acetimidate.

After dialysis of the enzyme against 50 mM Hepes, 500 mM NaCl and 0.5 mM DTT (pH 8.0), 0.5 ml methyl acetimidate solution (40 mg/ml in 1 M Na_2CO_3 + $NaHCO_3$ buffer pH = 9.2) was added to 0.5 ml enzyme solution (0.6 mg protein) and incubated at 4°C for 8 to 10 hours. Due to H^+ liberation during methyl acetimidation the pH is lowered to 8.0. For the determination of rates of lysine modification

aliquots were dialyzed against 3x3 L buffer (25 mM Hepes, 150 mM NaCl, 1.5 mM DTT, pH 8.0), each dialysis lasting 3 h at 4°C. Amino end groups of the enzyme protein were assayed with the ^{125}I-labeled Bolton-Hunter reagent as follows. To 25 µg enzyme protein (24.5 pmol NH_2 groups) 510 pmol ^{125}Bolton-Hunter reagent were added (2.7 x 10^7 cpm) incubated overnight (at 4°C), mixed with 20% TCA, further incubated for 1 h and the precipate filtered onto GF/C (Whatman, filter paper, washed with 20% TCA (10x2 ml) and with 3 x 2 ml ETOH and ^{125}I counted. Under these conditions 24.9 pmol ^{125}I-Bolton-Hunter reagent bound to the given amount of enzyme protein (102%), while after methyl acetimidate treatment for 10 h this was reduced to 1.5 pmol (6%) indicating that 94% of all lysine residues were modified.

Covalent binding of the enzyme protein to Affigel 10 matrix.

After dialysis of the enzyme against 25 mM Hepes, 250 mM NaCl and 2 mM DTT buffer (pH 8.0), 100 µg enzyme protein (containing 350 µg bovine serum albumin and 10 µg/ml coenzymic DNA in a volume of 2 ml) was added to 1 ml of packed Affigel 10, that had been previously washed with the above buffer and incubated for 4 hours with shaking at 4°C. The reaction was quenched with 1 ml 2 M TRIS-HCl, (pH 7.4) buffer and the incubation continued for one additional hour. The gel containing 150 µg protein (equivalent to 33 µg enzyme) per ml was washed with the above buffer solution (the concentration of NaCl was raised to 0.5 M in this buffer) and finally with 150 mM Tris, 10 mM $MgCl_2$, 2 mM DTT (pH 8.0). The matrix bound enzyme catalyzed ADP-ribose incorporation with a modified kinetics (Table I) but was unstable even in the frozen state ($t_{1/2}$=1 week).

Binding of the native and methyl acetimidate modified ^{125}I-labeled poly (ADP-ribose) polymerase to DNA cellulose (16).

Varying concentrations of enzyme protein (from 0 to 78 nM) were incubated with a constant amount of DNA-cellulose (7 µg DNA-equivalent) in 250 µl 100 mM Tris-HCl,

10 mM $MgCl_2$, 2 mM DTT, (pH 8.0), containing 100 µg/ml bovine serum albumin, for 20 min. at 25°C. Non specific binding was assayed by quenching with 250 µg/ml calf thymus DNA. At the end of incubation the resin was sedimented for 4 min. at 2500xg and the ^{125}I-labeled enzyme determined in the supernatant. Bound and free enzyme concentrations were calculated and their relationship determined by Scatchard plots.

Iodination of the enzyme protein.

Ten to 20 µg of IODO-GEN (Pierce, 1,3,4,6-tetrachloro-3,6,-diphenylglycouril) was dissolved in 200 µl $CHCl_3$ and the solvent evaporated, leaving a film on the surface of the tube. Two hundred µg enzyme protein, dissolved in 133 µl of 100 mM TRIS-HCl (pH 7.4) 150 mM NaCl, was added to the tubes and incubated for 10 min. at 4°C with 1 mCi of ^{125}I-NaI. The protein was separated from the unreacted ^{125}I by gel filtration on a Sephadex G75 column (0.7 x 6 cm), previously equilibrated with the same buffer supplemented with 2 mM DTT. More than 90% of the radioactivity was acid precipitable and the enzyme fully retained its catalytic activity after iodination. The specific radioactivity was 107 cpm/fmol for the native enzyme, and 84 cpm/fmol for the methyl acetimidate modified enzyme.

Separation of mono - and poly (ADP-ribose) by high performance liquid chromatography (HPLC).

The auto poly-ADP-ribosylation of the poly (ADP-ribose) polymerase enzyme was stopped by 5 mM benzamide and mono and oligo ADP-ribose were determined in base hydrolysates as reported (14).

Determination of the average chain length of poly (ADP-ribose).

The poly (ADP-ribose) containing HPLC fractions (eluted with buffer C cf. 14) were dialysed against several changes of water, (pH set to 8 - 8.5 with Tris Base) freeze dried and dissolved in 50 mM Tris pH 7.6, 5 mM $MgCl_2$. To 100 µl aliquot, 0.5 U of snake venom phosphodiesterase (freed from phosphatase by heat treatment) was added and incubated for 1 hour at 37°C, followed by digestion with 50 µg of proteinase K for 30 min. and AMP and phosphoribosyl-AMP determined by TLC as described above. The average chain length n was calculated as $n = \frac{[AMP] + [PR\text{-}AMP]}{[AMP]}$.

Assay of hydroxylamine sensitive and resistant poly (ADP-ribose) protein bonds (15).

Native (25 µg) or methylacetimidate modified enzyme (50 µg) was reacted with 34 µM ^{14}C-NAD for one hour at 25°C in 250 µl buffer (see Enzymatic Methods). The native enzyme system contained also 10 µg/ml coenzymic DNA. At the end of the reaction 250 µg of bovine serum albumin was added, and proteins were precipitated with 10% of TCA at 4°C for 15 min. The precipitates were suspended in 500 µl of 100 mM TRIS-HCl pH 7.0 or in 500 µl of 1.0 M Hydroxylamine pH 7.0 with sonication and incubated with shaking at 25°C. The hydroxylamine induced first order decay of protein polymer adducts was determined by the decrease of acid precipitable protein bound polymers.

Results

Figure 1. illustrates the rate of inactivation of the DNA dependent polymerase activity of the enzyme, which follows a typical first order Kinetics ($t_{½}$=0.518 h, $K^I = 1.34\ h^{-1}$). The consequences of this enzyme modification were as follows.

Figure 2. shows the decrease of the binding of the lysine modified enzyme to DNA cellulose as compared to the native enzyme (both labeled with ^{125}I) from K_D = 15.7 $\pm$ 1 to K_D 47.8 $\pm$ 2 nM, demonstrating that intact lysine residues are required for the binding of coenzymic DNA to the enzyme protein. Since the methyl acetimidate addition to lysine does not remove the positive charge of N (12) the DNA binding to lysine must be more specific than electrostatic attraction.

Figure 3. illustrates that the modified enzyme can still serve as polymer acceptor, when incubated with native polymerase, and the quantity of polymer protein adducts is proportional to the amount of lysine modified enzyme protein until saturation is reached. The conversion of the polymerase to mono ADP-ribose transferase is shown in Table I where products were determined after 1 h incubation. Experiment no. 1. indicates the mono and poly (ADP-R) formation without added DNA and Experiment no. 2 the effect of DNA on the native enzyme, promoting elongation. As shown in Experiment no. 3 the blocking of lysine end groups results in a drastic decrease in polymer but little change in monomer formation. In Experiment no. 4 the matrix bound polymerase was incubated with the lysine modified enzyme, and the enzymatic reaction terminated by reisolation of the modified free protein. The products formed on the modified protein were analyzed. Similar to No 3, in No. 4 the formation of monomer adducts was close to the rate catalyzed by the native enzyme without DNA (No.1) but the oligomer synthesis was low. The matrix bound enzyme alone with added DNA synthesized relatively small quantities of polymers (No. 5), demonstrating that the physical state of the enzyme itself can modify the nature of products.

When initial rates (in 1 minute) of poly ADP-ribosylation and of the extra hydrolysis of NAD (NAD-glycohydrolase activity) were compared results in Table II

were obtained. As in long term experiments (Table I) coenzymic DNA increased rates of poly ADP-ribosylation of the native enzyme 4.7 fold (Experiments nos. 1 and 2) whereas NAD-glycohydrolase activity was only doubled (Experiments 3 and 4). The lysine modified enzyme displayed poly ADP-ribose polymerase activitiy which was of the same magnitude as for the native enzyme without added coenzymic DNA. This enzymatic activity of the modified enzyme was unaffected by DNA, demonstrating the primary requirement of free lysine residues for the elongating coenzymic function of DNA (Experiments nos. 5 and 6). Similar results were obtained with NAD-glycohydrolase activity, which was lower in the modifed enzyme than in the native enzyme, but unaffected by coenzymic DNA (Experiments nos. 7 and 8).

The fraction of hydroxylamine stable and unstable protein-ADP-ribose bonds approximately agreed with reported values (15), indicationg that about 40-45% ester bonds existed in both the native and lysine modified enzyme. The first order decay rates of ester bonds (K^I_{native} = 1.04 h^{-1}, $K^I_{modified}$ = 1.15 h^{-1}) were nearly identical in both the native and modified proteins. The total amounts of hydroxylamine unstable ADP-ribose protein bonds were estimated from plateau values (after 45 min reaction). These results can be explained as follows. One mg enzyme (8 nmol) contains 7 polymer initiating sites without added DNA and 15 sites in the presence of coenzymic DNA (calculated from Table I). After the reaction with methyl-acetimidate (94% completion) there are still about 6 free lysine residues left, capable of forming Schiff base type adducts with ADP-ribose (10) that is generated by the NAD-glycohydrolase activity of the enzyme (calculated from Table II), sufficient to account for the 40-50% hydroxylamine insensitive bonds, in agreement with analytical values.

Discussion

Specific modification of lysine end groups of poly ADP-ribose polymerase inhibited the DNA dependent polymer synthesis coincidental with a diminution of DNA binding, without drastically affecting NAD-glycohydrolase activity or the auto-mono-ADP-ribosylation of the enzyme protein. These results for the first time identify the coenzymic DNA as an elongation co-factor. Its mode of action depends on the binding of DNA to the polymerase protein and we assume that the synthesis of oligomers may involve some form of DNA-dependent conformational alteration of the polymerase. In support of this hypothesis preliminary results indicate a co-operative catalytic action of DNA.

The reported model experiments predict that alkylating drugs hitherto thought to affect the process of ADP-ribose transfer from NAD to acceptor proteins exclusively by acting on DNA (17, 18) may have an alternative, direct catalytic effect on the polymerase enzyme protein itself, similar to the action of methyl acetimidate. This hypothesis is made plausible by the primarily surface localization of lysine residues in globular proteins (19), predisposing them for reactions with drugs or physiological regulatory molecules. Acetylation and deacetylation of lysine residues may serve as physiological regulators of the polymeric chain length of poly ADP-ribose within chromatin.

The dual catalytic action of poly ADP-ribose polymerase can provide a mechanism for the thus far unexplained generation of hydroxylamine sensitive and insensitive ADP-ribose protein bonds. The already documented enzymatically synthesized ester type bond formation between ADP-ribose and Histone H_1 (20) may be generally applicable to other ADP-ribosylated proteins, whereas the non-ester type bonds could be due to Schiff base protein-ADP-ribose elongation templates (11) produced by the NAD glycohydrolase activity of the polymerase. Consistent with this mechanism is the polymer initiating and acceptor propensity of the lysine modified enzyme protein that can serve as acceptor substrate to the fully active native polymerase.

References

1) Hayaishi O. and Ueda K. (1977) Ann Rev Biochem 46, 95-116

2) Hayaishi O. and Ueda K. (eds) ADP-ribosylation Reactions, Biology and Medicine (1982) Mol Biol Series Acad Press, New York

3) Pekala P.H. and Moss J. (1983) Curr Topics Cell. Regul. (Eds Horecker B.L., and Stadtman E.) Acad Press 22, 1-43

4) Yoshihara K., Hashida T., Tanaka Y., Ohgushi H., Yoshihara H. and Kamiya T., (1978) J. Biol Chem 263, 6459-6466

5) Kawaichi M., Ueda K, and Hayaishi O. (1981) J Biol Chem 256, 9483-9489

6) Miwa M., Saikawa N., Yamaizumi A., Mishimura S. and Sugimura T., (1979) Proc Natl Acad Sci USA 76, 595-599

7) Minaga T. and Kun E. (1983) J Biol Chem 258, 725-730

8) Minaga T. and Kun E. (1983) J. Biol Chem 258, 5726-5730

9) Kun E., Kirsten E., Milo G.E., Kurian P and Kumari H.L. (1983) Proc Natl Acad Sci USA 80, 7219-7223

10) Kun E., Chang A.C.Y., Sharma M.C., Ferro A.M. and Nitecki D. (1976) Proc Natl Acad Sci USA 73, 3131-3135

11) Ueda K., Kawaichi M., Okayama H. and Hayaishi O. (1979) J Biol Chem 254, 679-687

12) Hunter M. and Ludwig M.L. (1972) Meth. In Enzymology XXV 585-596

13) Ho S., Shizuta Y. and Hayaishi O., (1978) J Biol Chem 258, 3647-3651

14) Hakam A., McLick J. and Kun E. (1984) J. Chromatography 296, 369-377

15) Adamietz P., Wielckens K., Bredehorst R., Lengyel M. and Hilz H. (1981) Biochem Biophys. Res. Commun. 101, 96-103

16) Alberts, B. and Herrick G. (1971) Meth. Enzymology XXI. (Eds. Grossman, M.J. and Moldave, K.) Acad Press NY 198-217

17) Durkacz, B.W., Irwin J. and Shall, S. (1981) Eur. J. Biochem. 121, 65-69

18) Durkacz, B.W., Omidiji, O., Gray, D.A. and Shall, S. (1980) Nature 258, 593-596

19) Stryer, L. (1968) Ann Rev Biochem 37, 25-50

20) Ogata, N., Ueda K. and Hayaishi, O. (1980) J Biol Chem 255, 7610-7620

Table I

Enzymatic products formed by native and modified poly(ADP-ribose) polymerase and the effects of coenzymic DNA

No.		ADP-ribosylation (n mol/mg protein/hour)		Average chain length of oligomers
		mono ADP-ribose	poly ADP-ribose	
1.	poly(ADP-ribose) polymerase activity in the absence of DNA	41.6	9.96	6.1
2.	poly(ADP-ribose) polymerase activity in the presence of DNA	86.2	459.7	52.0
3.	methyl acetimidate modified poly(ADP-ribose) polymerase without DNA	26.3	0.89	2.9
4.	acceptor properties of modified enzyme protein incubated with DNA and matrix bound enzyme	37.6	2.2	3.9
5.	auto ADP-ribosylation of the matrix bound poly (ADP-ribose) polymerase in the presence of DNA	6.9	13.8	14.2

TABLE II

The effects of lysine modification on the polymerase and NAD-glycohydrolase activities of the poly (ADP-ribose) polymerase enzyme protein.

NO.	EXPERIMENTAL CONDITIONS	ACTIVITY(nmol/min per mg PROTEIN	ACTIVITY + DNA / ACTIVITY - DNA
1.	native polymerase -DNA	33.9	-
2.	native polymerase +DNA	158.5	4.7
3.	native glycohydrolase -DNA	18.9	-
4.	native glycohydrolase +DNA	40.2	2.1
5.	modified polymerase -DNA	21.2	-
6.	modified polymerase +DNA	23.08	1.1
7.	modified glycohydrolase -DNA	3.8	-
8.	modified glycohydrolase +DNA	3.9	1.0

Figure I

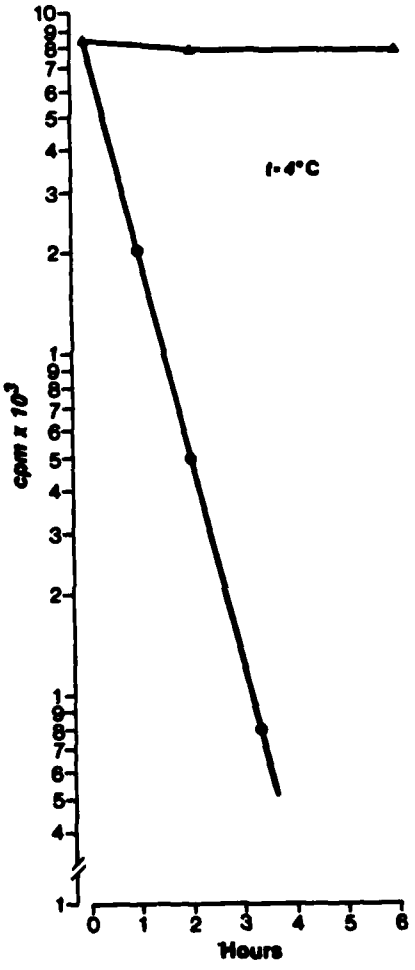


Figure 2

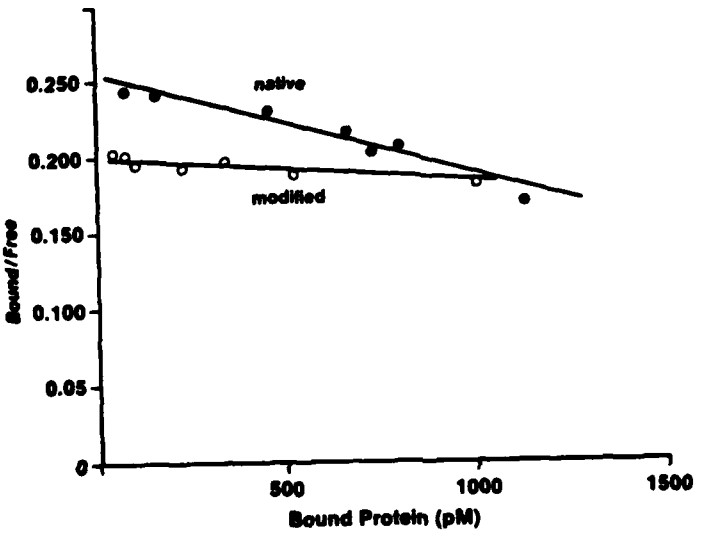
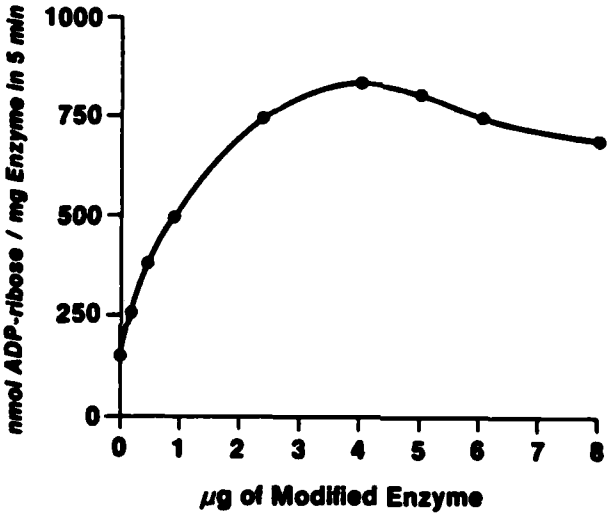

native
modified
0.250
0.200
0.150
0.100
0.05
0
Bound/Free
500
1000
1500
Bound Protein (pM)

Figure 3


nmol ADP-ribose / mg Enzyme in 5 min
1000
750
500
250
0
0
1
2
3
4
5
6
7
8
μg of Modified Enzyme

Legends

Table I. Each experiment (1 to 5) is a representative of 3 independent tests, which had a variance of ± 10-15%. In Experiment 1, 10 μg polymerase and 34 μM NAD; in Experiment 2, 5 μg polymerase, 10 μg/ml coenzymic DNA and 42 μM NAD; in Experiment 3, 25 μg polymerase and 34 μM NAD; in Experiment 4 and 5, 12.5 μg modified polymerase, 2.5 μg Affigel 10 matrix bound native polymerase, 10 μg/ml coenzymic DNA and 34 μM NAD were incubated in systems described in Experimental Procedures for 1 h at 25°C. In Experiment 3, the reaction was terminated by elution of the ADP-ribosylated modified polymerase with 3x2 ml 0.5 M NaCl. Mono and polymers were isolated and quantitatively determined as described in Experimental Procedures.

Table II. Results are representative of 3 independent series, as described in the legend of Table I. 2.5 μg native polymerase or 5 μg modified enzyme protein were incubated with 32 μM NAD in the presence and absence of 10 μg/ml coenzymic DNA for 0, 1.5 and 3 minutes at 25°C and both polymerase and NAD-glycohydrolase activites assyed as described in Experimental Procedures. Rates represent initial velocities.

Figure 1. Rate of inactivation of poly ADP-ribose polymerase by incubation with methyl-acetimidate (for enzyme assays, see Experimental Procedures). 25 μl enzyme (30 μg) and 25 μl methyl acetimidate (40 μg/ml) were incubated at 4°C in 1 M Na_2CO_3 - Na_2HCO_3 (pH at the end of reaction = 8.0) and 5 μl aliquots were withdrawn for polymerase assays. Rate constant was calculated from $\frac{0.693}{t_{\frac{1}{2}}}$

—●—●— incubation with methyl acetimidate

—△—△— control (containing HCl in equivalent amount to $[H^+]$ calculated to be formed from methyl acetimidate, to obtain pH 8.0)

Figure 2. Binding of ^{125}I labeled native and modified polymerase enzyme to DNA-cellulose. Technical details are described in Experimental Procedures.

Figure 3. Poly ADP-ribosylation (ordinate) of methyl acetimidate modified enzyme (varying concentration, see abscissa) by 0.5 μg native enzyme, as described in Experimental Procedures. The time of incubation was 5 minutes at 25°C.

IV

Cell transformation and poly(ADP-ribose)

This section contains research that commenced as collaboration with Ohio State University, a work which lead to papers number 1 and number 7. Because these papers appeared in print only a brief summary will be given. The following report contains also experiments concerned with UV-radiation induced cell transformation and its prevention by benzamide (by G.E. Milo, S. D'Ambrosio and E. Kun as collaborators). The initial collaborative cell biological work is presently continued and expanded in the Surge laboratories at UCSF (see Abstracts No. 1,3) by the research team whose work is presently summarized in this progress report. The UCSF lab is now fully functional in cell biology after installation of its own cell culture facilities.

Summary of papers Number 1 and 7

It was shown that cellular transformation by ultimate carcinogens is S phase specific (ref. 1.) and prevention of transformation by benzamide (ref. 1.) or 12 other molecules that bind to the poly(ADP-ribose) polymerase system (ref. 7.) is equally restricted to S phase. More importantly exposure to antitransforming drugs alone confers apparent resistance to transformation inducing agents 6 generation later, indicating that an "inheritable" genetic propensity has been induced by the binding of antitransformers to the presumably coenzymic (to poly(ADP-ribose) polymerase) DNA segment. (see ref. 10, 11.)

Conclusions and predictions

These basic observations are in good agreement with current concepts of neoplastic transformation by the following arguments:

1. S phase is known to express certain proto-oncogenes (C-fos and myc genes) in a reversible manner. This explains S phase specificity of "transformability." Presumably in transformed cells expression of proto-oncogenes will persist.

2. Our current work focuses on the identification of the DNA segment which is responsible for: a.) poly(ADP-ribose) polymerase binding and b.) "promotor" function. This "promotor" DNA segment if modified (e.g. by antitransformers) may allow proto-oncogenes to remain functioning as "normal" genes. Our present hypothesis is that a.) and b.) are either identical or closely related, providing a genetic molecular explanation of phenotypic control via this critical DNA sequence(s?). Clearly this conclusion and prediction transgresses the boundaries of the present report and forecasts our future plans. Similarly the inducibility of poly(ADP-ribose) polymerase (ref. 3) also is a connecting link to new research areas (see grant application concerned with the isolation of the polymerase gene).

UV-radiation induced cell transformation, its prevention by benzamide, and the irrelevance of DNA breakage and repair to transformation.

Summary

Synchronized human fibroblasts were exposed in early S phase to increasing doses of UV-irradiation in presence and absence of an antitransforming drug, benzamide. Cellular survival, initial thymidine dimer formation and cellular phenotypic transformation were simultaneously determined in the presence and absence of 1 mM externally added benzamide. Cellular transformation was the only process that was inhibited by the non-toxic dose of benzamide, whereas all other parameters remained uninfluenced by the antitransforming drug. These results suggest that thymidine dimer formation and its repair are unrelated to the process of transformation.

When synchronized human fibroblasts are exposed in the early S phase to rigorously defined non toxic concentrations of ultimate carcinogens cellular transformation occurs that can be prevented by equally non toxic concentrations of benzamide presented to cells simultaneously with the carcinogens (1). More recently a single exposure of cells to benzamide without carcinogens was shown to result in the development of carcinogen resistance (18). Anti-transforming effects of non toxic concentrations of 3-amino benzamide against carcinogens and radiation were subsequently reported in C3H10T½ mouse embryo cells (2,3). In contrast to these results when high doses of inhibitors of poly (ADP-ribose) polymerase were administered in vivo, or added to cells in culture, presumably sufficient to inhibit this enzyme, both the toxic and carcinogenic effects of high doses of carcinogens (4) and alkylating drugs (5,6) were augmented, demonstrating the critical drug dose dependence of these phenomena. The increase in rates of poly ADP ribosylation following treatment with carcinogens has been ascribed to a correlation with DNA damage and repair (7,8, for review 24) but molecular details are as yet unknown. Ultraviolet radiation produced an increase in poly ADP-ribosylation and simultaneous augmentation of DNA strand breaks in human fibroblasts (9, 24). However, the inhibition of poly ADP-ribosylation by 3 mM aminobenzamide did not alter DNA-repair replication that was induced by UV radiation (9), therefore, it appears difficult to discriminate between coincidental or causal correclations.

In the present report we have analyzed in synchronized human fibroblasts a well documented effect of UV radiation on DNA, thymidine dimer formation and its repair (10, 11), and attempted to correlate this with UV radiation induced cellular transformation, and its prevention by a non toxic concentration of benzamide.

MATERIALS AND METHODS

Culture Dynamics for G_1 Block and S Period

Human neonatal skin fibroblasts(HNSF)were prepared and cultured in Dulbecco's modified minimum essential medium (MEM) as previously reported (12). These logarithmically growing cultures have a radiolabelling index of 18-22% at a 25-30% confluent density (1:4 plating density). The cells were blocked in G_1 by removing the MEM, washing the cultures twice with arginine and glutamine deficient MEM and refeeding them with this medium supplemented with dialyzed fetal bovine serum (13). After the cells were in the G_1 block for 24 hours the medium was removed and the cells were washed and refed with MEM containing glutamine and arginine, 10% fetal bovine serum and 0.5 units of insulin per ml. These cells entered S circa 10 hours later. The cells were treated as decribed below.

Cell Treatment

At the onset of the S phase of the cell cycle, 1nM benzamide (final concentration) was added or not added to the cell cultures. Three hours later the medium was removed and the cell cultures were washed twice with warm (37°) Hanks Balanced salt solution. This solution was removed and the cells were exposed to 0 to 13.2 J/m^2 UV radiation emitted from a 15W germicidal lamp (15GT8) at a fluence rate of 1.1 J/m^2 sec. Immediately following the UV irradiation, fresh warm (37°) growth medium with or without 1 mM benzamide was added. The cultures were incubated at 37° and used as described below for the determination of toxicity, anchorage independent growth or UV-endonuclease sensitive sites in DNA. The experimental design of cell treatments is shown in Fig. 1.

Cellular Toxicity and Determination of Anchorage Independent Growth

At the conclusion of the S phase of the cell cycle, cells were serially passaged 1:2 into an eriched growth medium (1,13). For toxicity determination, 200 to 1000 cells were plated into Corning 60 mm diameter tissue culture dishes as described previously (1, 14) The medium was replaced 3 times each week until colonies were visible under 10x phase contrast microscopy in approximately 2 to 4 weeks. The cells were fixed, stained and counted (1,14).

Anchorage independent growth was determined by growth in soft agar (1,14). Briefly, cells were serially passaged at 1:10 split ratio until 16 to 20 population doublings. At this time, 5×10^4 cells were seeded into soft agar and allowed to grow. The number of colonies formed were determined following fixation and staining (1,13). Cells were also evaluated for the presence of a sarcoma associated cell surface antigenic determinant and cellular invasiveness as reported previously (1). The selection procedures used for HNSF were compared with fibrosarcoma, leiomyosarcoma and synovial sarcoma cells.

UV-Endonuclease Sensitive Site Assay

HNSF were incubated with 2 μCi/ml [^{3}H]-thymidine (S.A. 6.7 mCi/mmole) or [^{14}C]-thymidine (S.A. 450 μCi/mmole) for 48 hours as described previously (11,12). The labeled cells were removed from the surface of the plates with trypsin and seeded into 60 mm Corning tissue culture dishes at a density of 3×10^3 cells/cm^2. After the cells had attached to the surface of the plates, the medium was changed and the cells synchronized. The ^{3}H-labeled cells were incubated with benzamide as described above for cell transformation. Immediately before irradiation, the medium was removed and the cells washed twice with phosphate buffered saline. Cells were irradiated (12) and immediately following irradiation, a set of the ^{3}H- and ^{14}C-labeled cells were removed and

the DNA extracted. This DNA extract was used for the determination of the total number of dimers induced by UV radiation. To the remaining tritium prelabeled cells, fresh growth medium containing benzamide was added. Only fresh growth medium was added to the remaining ^{14}C-labeled cells. Both sets of cultures were incubated at 37°C for 2 hours. At the indicated times, the medium was removed and the cells washed twice with 1 ml of an ice-cold salt-EDTA solution (0.1 M NaCl, 0.5 mM EDTA, 2.7 mM KCl, 1.5 mM KH_2PO_4 and 8.1 mM Na_2HPO_4). The ^{3}H-labeled cells were combined with the ^{14}C-labeled cells to yield 2000 cpm of each isotope. The DNA was extracted using the phenol method and incubated at 37°C for 1 hour with pyrimidine dimer-specific endonuclease isolated (DE-52 chromatography) from Micrococcus luteus as reported previously (10, 11). The number of pyrmidine dimers was calculated from the number average molecular weight (1/Mn) following centrifugation of the DNA through 5 to 20% alkaline sucrose gradients in (0.5M NaCl) for 90 min at 45,000 rpm in a SW 50.1 rotor of a Beckman L5-55 ultracentrifuge, as described (15).

Results

Cellular Transformation and Survival

The effects of UV radiation in early S phase are shown in Table I. At a non toxic UV dosage significant cell transformation was apparent (Expt. 2) and benzamide prevented it by 97%. Increasingly toxic radiation, as detected by a decrease of colony formation, also induced transformed cells. Benzamide completely inhibited the development of the transformed phenotype (Expt. 3, 4). The nature of transformed cells was further studied by their isolation. Cells of anchorage independent colonies exhibited the human malignancy specific cell surface antigenic determinant (1) and when inoculated into nude mice (16) developed tumors. Their relatively low incidence (3-5/10) is probably

explained by lack of syngeny (cf. 1.). Histological examination of tumors identified them as proliferating myxofribroma cell types. It is noteworthy that tumor incidence of human fibrosarcoma, leiomyosarcoma and synovial sarcoma cells in nude mice was identical with that of UV-transformed human fibroblast. The life span of transformed cells was ca. 120 PDL compared with 35± PDL of normal fibroblast. Several human sarcomas showed also a limited life span in cultures, close to the UV-transformed fibroblasts.

Thymidine dimer formation and DNA repair.

Alkaline sucrose gradient profiles (see UV-endonuclease sensitive sites, in Materials and Methods) of DNA extracted from unirradiated (Fig. 1, A) and UV irradiated cells (3.3 J/m^2) were compared in the presence and absence of benzamide (Fig.1, B). The shift of DNA profile (compare A and B) indicates the change in the molecular mass of DNA, as a result of the UV-endonuclease digestion at the thymidine dimer sites (see Methods). Approximately 4.7 thymidine dimers per 10^8 Dalton DNA are induced by 3.3 J/m^2 UV radiation and this number was uninfluenced by benzamide (B). In the experiment shown in Fig. 1, C cells were incubated for 2 hours following UV irradiation (3.3 J/m^2) in the presence and absence of benzamide, to allow DNA repair. Repair was about 64% in both cases (Table 2)which was uninfluenced by benzamide. The process of thymidine dimer formation, as determined by the decrease of the molecular mass of DNA, and repair during 2 h after irradiation are summarized in Table 2. It is apparent that none of these processes were influenced by benzamide whereas transformation was inhibited under the same conditions.

Discussion

It has been reported that fixation of a signal leading to cellular transformation requires DNA synthesis and cell replication (17), and the S phase specificity of the transforming effect of ultimate carcinogens, applied at non toxic doses (1) agrees with this conclusion. The coincidental antitransforming effect of benzamide, exhibiting early S phase specificity (1) suggests that both transforming drugs and benzamide, appear to act on the same, as yet unidentified site. Recent studies(18) indicate that brief exposure of human fibroblasts in early S can actually produce cells that are resistant to transformers added to the cells 6 PDL later. Therefore, molecular events induced by 6-10 μM intranuclear concentration of benzamide (cf.1) seem to alter phenotypic response for several generations. The nature of these nuclear events are as yet unknown but they may be related to the selective binding of benzamide to the coenzymic DNA of poly(ADP-ribose) polymerase in cell nuclei (19). Since the intracellular concentration of benzamide in various cell types at high extracellular drug and carcinogen levels (4,5), have not been determined, it seems impossible to interpret the mode of action of these high doses of drugs, producing cellular toxicity (4,5,8,9,24), the latter probably obscuring selective effects that only occur at non toxic concentrations of drugs. The complicating effect of non specificity of action of high concentration of benzamides has also been emphasized by others (20). It is apparent that thymidine dimer formation by relatively non toxic UV radiation and DNA repair shows no correlation with the antitransforming effect of benzamide. An analogous conclusion has been drawn for the DNA-damaging effects of carcinogens that could not be correlated with the antitransforming action of benzamides in C3H10T½ mouse fibroblasts (cf. 2).

Contrary to our experience (1, 12, 13, 14, 16, 18, 21), it has been stated that successful carcinogenic initiation may occur at any stage of the cell cycle (22).

It seems to us that evaluation of this conclusion is complicated by the large differences in time constants between rapid DNA modifying reactions and much slower cell cycle kinetics and these large discrepancies (in $t_{\frac{1}{2}}$) would make it impossible to locate rate limiting events of transformation in a random cell population. On the other hand the ineffectivity of the antitransforming action of benzamide in other phases than early S (1) tends to locate the time of critical transforming events induced either by carcinogens or UV.

The relevance of our results to cancer is suggestive, but uncertainties in this area prohibit exact evaluation. Chemically or UV-transformed human fibroblasts do not exhibit immortality or unrestrained invasiveness in different species, similar to several types of human cancer cells isolated from surgical material (12, 16). However, these phenomena may be expressions of as yet undefined stages of transformation. We have shown in unpublished work that chemical transformation of 3T3 (NIH) cells to transformants that produce lethal tumors in nude mice can be blocked by benzamide or similar acting drugs (18), just like the transformation of human fibroblasts, therefore the basic processes of transformation and inhibition by benzamide may be similar regardless of the resulting ultimate cellular phenotypes. It is possible that some form of alteration of DNA rearrangement, induced by the binding of benzamide to DNA (19), may be the molecular basis for the antitransforming effect of this drug. A benzamide-induced apparent translocation of DNA-benzpyrene-diol-epoxide adduct from core to linker region (23) tends to support this type of mechanism.

Table I

The effects of UV radiation and benzamide on cellular survival and on colony formation of transformed cells determined by anchorage independent growth.

Experiment No.	UV (J/m^2)	% Relative Colony Formation	Colony Formation in Soft Agar per 10^5 Seeded Cells	
			-BA	+BA
1	0.0	100	0	0
2	3.3	100	105 ± 25	3 + 2
3	6.6	75	37 + 13	0
4	13.2	25	1.2 ± 1.4	0

n = 8 for experiments in the absence of benzamide; n = 12 for experiments where the effects of UV and benzamide were tested.

Experimental details of irradiation, drug treatment and determination of colony formation are described in Materials and Methods.

TABLE 2

EFFECT OF BENZAMIDE (BZ) UPON IRRADIATED DNA[1]

Sample		Number Average Molecular Wt.[2] (x 10^8 Da)		Number of Pyrimidine Dimers[3] (per 10^8 Da)		Percent Dimers Repaired	
UV (J/m^2)	Time after irradation (hr)	+BZ	-BZ	+BZ	-BZ	+BZ	-BZ
3.3	0	0.14	0.14	4.78	4.74	-	-
3.3	2	0.24	0.24	1.67	1.73	65.1	63.5

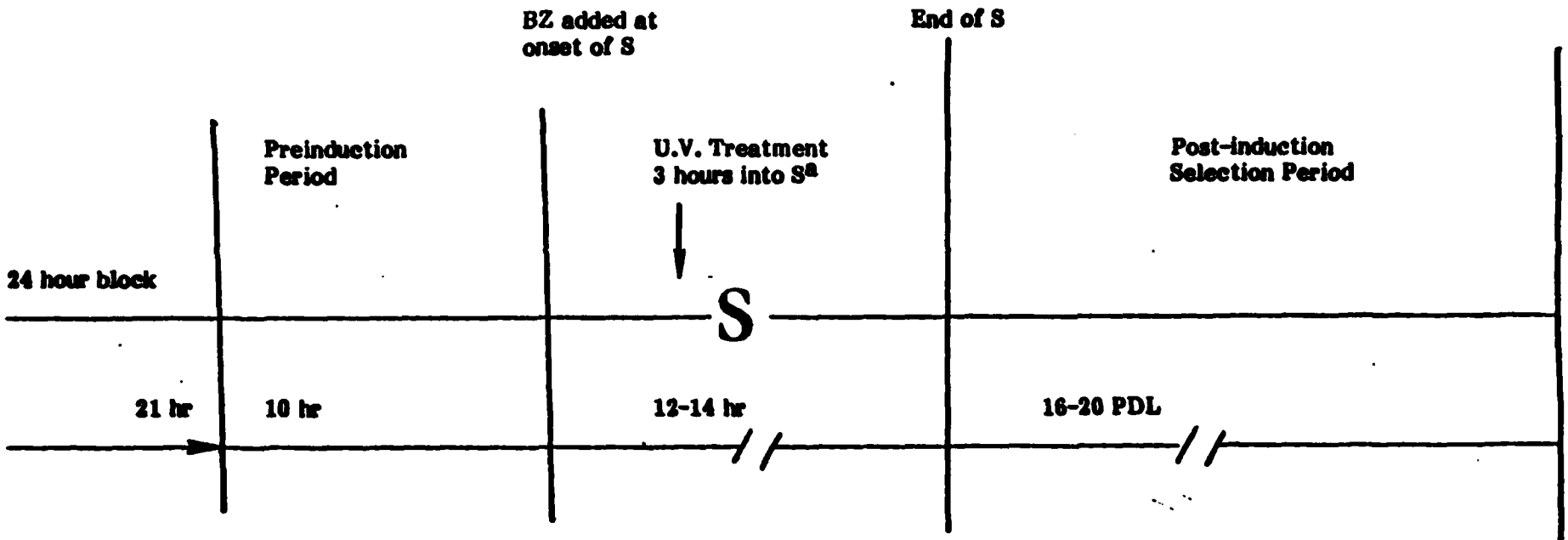
[1]Conditions for U.V. irradiation, sedimentation of DNA and calculation of Mn, dimers and percent repair are as described in Figure 1 and METHODS.

[2]The number average molecular weight for control cell DNA was 2.44 and 2.41 x 10^8 with and without BZ, respectively.

[3]Number of pyridimidine dimers = $1/Mn\ (DNA_{+UV\text{-}endo}) - 1/Mn\ (DNA_{no\ UV\text{-}endo})$ (see Methods)

FIGURE 1

PROGRAM OF U.V. INDUCED CELL TRANSFORMATION



[a]The phenol-red containing growth medium (MEM + 10% FBS) was removed and the cell sheet rinsed with HBSS. The U.V. exposure was carried out using a 15 W Germicidal Electric Lamp (15GT8) at a fluence rate of 1.1 $J/m^2/sec^2$. The fluence rate was measured by a Blak-Ray U.V. meter (U.V. Products, International, San Gabriel, CA). During the U.V. treatment the experimental medium containing the BZ was removed.

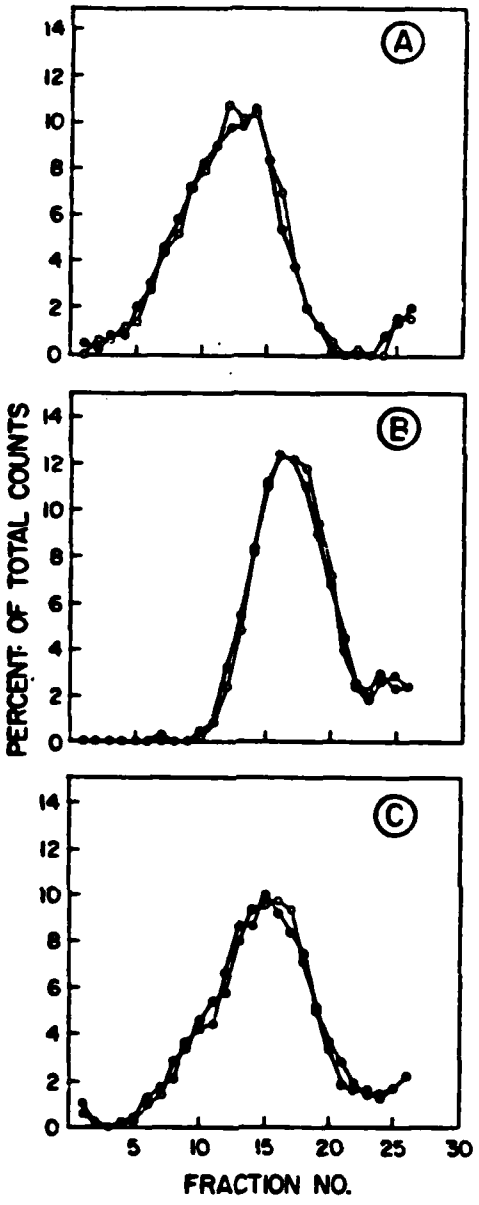


Fig. 2.

UV-endonuclease sensitive sites in DNA determined by alkaline sucrose density gradient profiles (see Results)

References

1. Kun, E., Kirsten, E., Mil, G.E., Kurian, P. and Kumari, H.L., Proc natl acad sci USA 80, (1983) 7219

2. Borek, C., Morgan, W.F., Ong, A. and Cleaver, J.E., Proc natl acad sci USA 81 (1984) 243

3. Borek, E., Ong, A., Morgan, W.F. and Cleaver, J.F., Radiation res 99 (1984) 219

4. Takahashi, S., Ohmishi, T., Denda, A. and Komishi, T., Chem biol interact. 30 (1982) 363

5. Lubet, R.A., McCarvill, J.T., Putnam, D.L., Schwartz, J.L. and Schechtman, L.M., Carcinogenesis 5 (1984) 459

6. Berger, N.A., Catino, D.A. and Vietti, T.Y., Cancer res 42 (1982) 4382

7. Durkacz, B.W., Omidiji, O., Gray, D.A. and Shall, S. Nature 283 (1980) 593

8. Durkacz, B.W., Irwin, J. and Shall, S., Eur j biochem 121 (1981) 65

9. Jacobson, E.L., Antol, K.M., Juarez-Salinas, H. and Jacobson, M.K. J. biol chem 258 (1983) 103

10. Gibson-D'Ambrosiom R.E., Leong, Y. and D'Anbrosiom S.M., Cancer res 43 (1983) 5846

11. Gibson, R.E. and D'Ambrosio, S.M., Photochem and Photobiol 35 (1982) 181

12. Riegner, D., McMichael, T., Berno, J. and Milo, G., Tissue culture assoc lab man 2 (1976) 273

13. Milo, G.E. and DiPaolo, J., Nature 275 (1978) 130

14. Milo, G.E., Weisbrode, S., Zimmerman, R. and McCloskey, chem-biol int. 36 (1981) 45

15. D'Ambrosio, S.M. and Setlow, R.B., DNA repair mechanisms vol. 9, (1982) 499 Academic Press, NY (1978)

16. Donhoe, J., Noyes, I. Milo, G.E. and Weisbrode, S., In vitro 18 (1982) 429

17. Borek, C. and Sachs, L. Proc natl acad sci USA 59 (1968) 83

18. Milo, G.E., Kurian, P., Kirsten, E and Kun, E., Febs lett 179 (1985) 332

19. Bauer, P.I., Hakam, A and Kun, E. Proc VIIth internat conference on ADP-ribosylation, Vitznau, Switzerland (Oct. 1984) in press

20. Milan, K. and Cleaver, J.E., Science 223 (1983) 589

21. Milo, G.E. Human carcinogenesis (1983) pp. 431-450. Academic Press, NY

22. Silinkas, K.C., Kateley, S., Tower, J., Maher, V. and McCormick, J., Cancer res 41 (1981) 1620)

23. Kurian, P., Jeffrey, A., Kun, E. and Milo, G.E., Fed proc 43 (1984) no. 1887

24. Shall, S., chapt. 9 in "Nucleic Acid Symp. 13 (1984) 143 (Eds: Collins, A., Downess, C.S and Johnson, R.T., I.R.L. Press, Oxford, Washington)

Research Staff

	UCSF AFOSR Grant supported	UCSF Collaborators not supported by AFOSR
1. UCSF	Hakam, A.	Tseng, A.
	Kirsten, E.	Brooks, M.
	Bauer, P.I.	
	Sadeghi, M.	
	McLick, J.	
	Peller, L.	

2. Collaborators outside UCSF

OSU	Milo, G
	D'Ambrosio, S.
Oklahoma Research Foundation	Griffin, M.
University of Freiburg (West Germany)	Decker, K.

END

FILMED

6-85

DTIC