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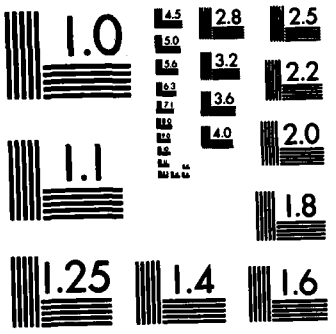

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SUPPLEMENTARY NOTES

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19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

Poly (ADP-ribose), precancerous state, prevention of carcinogenecity, hormone action

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

The patho-biological function of the nuclear homopolymer: poly ADP-ribose is being studied. It was found that this homopolymer, that has been identified recently by us to be a unique nucleic acid, with helical conformation, and is covalently bound to prevalently non histone chromatin proteins, exhibits specific quantitative signals during aging differentiation and precancerous pertubations.

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Preliminary evidence indicates that poly ADP-ribosylation of specific chromatin proteins by presumably modifying their binding to DNA, and regulating gene activity through an altered degree of poly ADP-ribosylation is part of the mode of action of deveopmental hormones (steroids, thyroid, growth hormones). In the precancerous state significant and specific increase in the poly ADP-ribosylation of a selected group of non histone proteins (160-200 kd) takes place, an effect that persists without detectable morphological cellular alterations.

The occurance of this phenonmenon was traced to the promoter phase of carcinogenesis as illustrated by the liver regeneration model. An equivalent cellular condition was identified in cell cultures at the G_1 block release stage that appears to provoke poly ADP-ribosylation increase, similar to the "promoter" pattern in the carcinogenic process in animal organs. In contrast to the carcinogenic process, in physiological stress induced hypertrophy and hyperplasia, and hormone induced growth, signal-specific and distinct responses in rates of poly ADP-ribosylation occur, consistent with a normally operative regulatory process involved in gene control. Present results show that determination of poly ADP-ribosylation of selected non histone proteins is a specific test for the precancerous state, permitting the identification of the molecular toxicology of agents that are suspected to be carcinogenic. The advantage to this detector system is its relative rapidity and specificity far exceeding conventional toxicological bioassays, predicting also a pharmacological method of cancer prevention applicable to real conditions of exposure to carcinogenic environmental conditions.

PROGRESS REPORT

October 1981-September 1982

"Molecular Toxicology of Chromatin"

(F49620-81-C-0007)

Principal Investigator

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University of California, San Francisco
Department of Pharmacology and CVRI

Date of Submission: November 15,1982

RESEARCH OBJECTIVES

Based on previous investigation in this laboratory that provided chemical and immunological methods of identification (see preceeding progress reports) of the hitherto little known nuclear homopolymer of ADP-ribose=poly ADP-ribose, the present investigations were concerned with the identification of the patho-biological function of this polymer in selected animal models. The pathologically meaningful model was= chemical carcinogenesis, the physiological models were age dependence and the action of thyroid, steroidal, and growth hormones. The involvement of poly ADP-ribosylation in hypertrophy, hyperplasia, and neoplasia has been demonstrated.

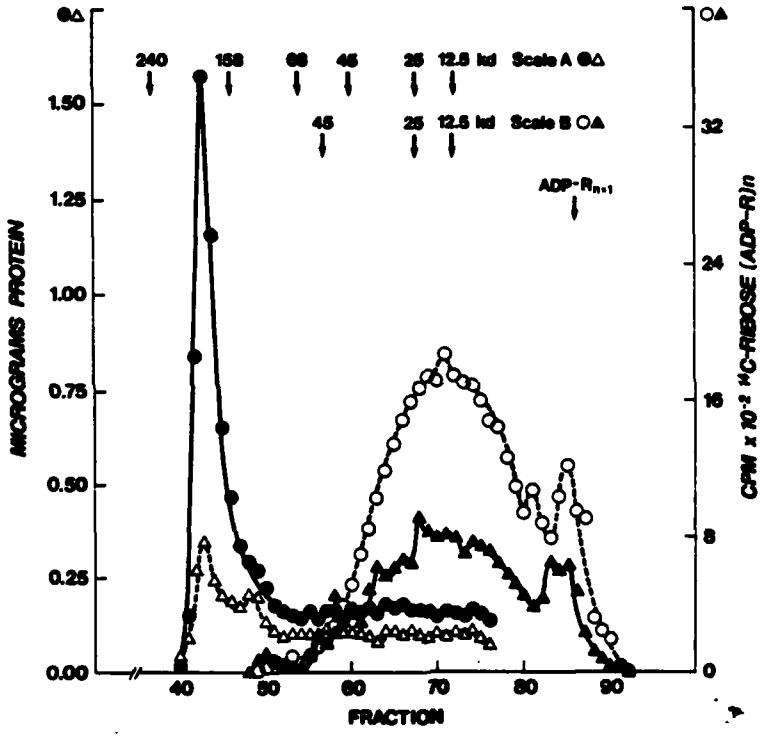
STATUS OF RESEARCH

I. Chemical Carcinogenesis and the Role of Poly ADP-ribose.

Poly ADP-ribosylated proteins were quantitatively isolated from livers of normal and dimethylnitrosamine treated Syrian hamsters by a novel affinity chromatographic method involving HPLC-molecular filtration. It was found that a selectively large molecular wt. group of non histone proteins (∿180-200 kd) were poly ADP-ribosylated at least 6-9 fold over controls, in livers of dimethylnitrosamine treated Syrian hamsters (ref. 1,2. The early phase of this work was

described also in the preceeding progress report). The important question emerged: is this increase of poly ADP-ribosylation of a selected group of non histone proteins specific for the precancerous state and what is the mechanism which leads to this increase.

Figure 1.



Legend: HPLC molecular filtration of poly ADP-ribosylated proteins (-●-, -△-△-) and isolated polymers (-▲-▲-, -○-○-). (cf ref 1).

KEY: -●-●- precancerous protein pattern
-○-○- precancerous polymer pattern

Two types of experimental questions were posed that were to elucidate this complex problem.

A.) Could the increase in protein-poly ADP-ribosylation in carcinogenesis be attributed to either the "promoter" or "initiator" conditions, two phases of the carcinogenic process that were originally defined about 30 years ago by Berenblum and Schubick.

A well documented promoter condition is liver regeneration, that can provoke hepatocarcinogenesis by minute doses of carcinogens which by themselves are ineffectual. We determined poly ADP-ribosylation in the regenerating liver (ref. 3) and found an increase of polymerase activity of the same magnitude as seen in carcinogen treated Syrian hamster livers (cf. 1,2). Thus, it was apparent that the promoter condition alone was contributory to the increase in poly ADP-ribosylation seen after treatment with the carcinogen (that itself causes cell regeneration following cell killing by the carcinogen in vivo thus providing both promoter and initiator conditions, (cf. 3). The initiator condition generally associated with the covalent binding of the carcinogen to DNA, and the increased poly ADP-ribosylation of selected non histone proteins (promotion)thus appeared to be a dual requirement for malignant transformation. Direct experimental determination of both stages of carcinogenesis is now feasible.

Although the final proof for the mechanism of this dual requirement was obtained only in the past 6 months, and further development of this critical work is part of the 1982-83 term of research, some significant information relevant to results reported

here will be summarized (see ref. 4).

In collaboration with Dr. G. Milo, we find that the G_1 block + release condition alone, a requirement for in vitro carcinogenesis in human cell cultures, produces biochemical signals in poly ADP-ribosylation that are the same as found in the regenerating liver (cf. 3). Consequently, the "G_1-block-release" condition and the "cell regeneration" in tissues are biologically equivalent precancerous, or promoter conditions, and are characterized by the increased poly ADP-ribosylation of the same set of (gene regulatory?) non histone proteins (unpublished results). Without the covalent binding of carcinogens to DNA this "precancerous" condition does-of course-not lead to cancer but-as will be reported elsewhere- (manuscript in preparation) the binding of carcinogens to DNA alone is equally insufficient to cause malignancy. The timed coincidence of both conditions are mandatory for successful malignant transformation. This is indicated by the fact that inhibition of poly ADP-ribosylation of the select group of proteins by an enzyme inhibitor (cf. 4) completely prevents carcinogenesis induced by a large variety of carcinogens, even though the inhibitor (of poly ADP-ribose polymerase) does not alter the covalent binding of the carcinogen to DNA (unpublished results).

Table I

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

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

** *Carcinogenesis was induced* by exposing human fibroblast after release from G_1 block for 10-14 hours to 1 μM carcinogens. The preventive effect of benzamide runs parallel with its inhibitory effect of poly (ADP-R) synthetase, which enzyme system is greatly increased at this stage. (cf. ref. 4)

II. Physiological Correlations

The second set of questions that are germane to the carcinogenesis related increase in poly ADP-ribosylation, are concerned with the problem: does poly ADP-ribosylation represent a physiological control system, and is this putative control system related to development and differentiation, the plausible physiological counterparts of malignant transformation? It would be predictable that carcinogenesis should reflect anomalies of these physiological processes. The

following experimental results provide an affirmative answer to these questions.

1. An age-dependence of poly ADP-ribosylation in selectively isolated cardiocyte nuclei has been found, indicating that within 3 weeks after birth, when DNA synthesis in cardiocytes stops, there is a simultaneous 10 fold drop in poly ADP-ribosylation in these nuclei (ref.5).

Figure 2. (cf ref. 5)

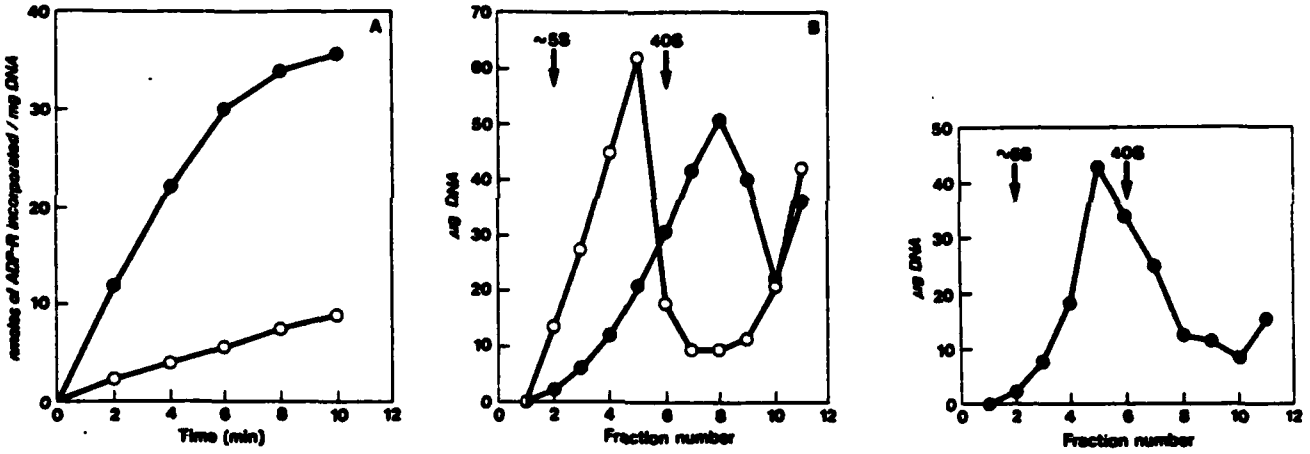


FIG. 1 (*left*). **Age-dependent changes in polyadenosine-diphosphoribose synthetase activity and of DNA size in cardiocyte nuclei.** Enzymatic activity (*ordinate*) was determined as described under "Experimental Procedures," and the time (*abscissa*) dependence of the reaction was followed for 10 min at 25 °C. In *A*: ●, enzymatic activity of cardiocyte nuclei of neonates; ○, that of adults. The typical experiment is one of 12 repeats. *B*, alkaline sucrose density ultracentrifugation profile of cardiocyte nuclear DNA of neonates (●) and of adults (○). The method was adapted from published techniques (17) using a 30-ml linear sucrose gradient (5–20%, w/v) with 5 ml of 60% (w/v) sucrose cushion, the former containing 0.4 M NaOH, 0.01 M EDTA, and 0.1 M NaCl (25). A lysis solution (0.5 ml) containing 0.5 M NaOH, 0.02 M EDTA, and 0.1% Nonidet P-40 was layered on top of the gradient followed by 0.5 ml of nuclear suspension containing 250 μg of DNA. After 15 min of lysis at 4 °C, centrifugation was carried out at 4 °C in a Beckman SW 28 rotor for 16 h with the aid of a Beckman L5-75 B ultracentrifuge at 27,000 rpm. After ultracentrifugation, fractions were collected from the top of each centrifuge tube in a Beckman fraction recovery system (Catalogue No. 339187) by forcing a solution of 60% (w/v) sucrose through the needle penetrating the bottom of the tubes. Then 3-ml fractions were collected, and macromolecular components were precipitated by an equal volume of 1 M $HClO_4$ and 4 mM EDTA. Nucleic acids and proteins in each fraction were separated as described earlier (9), and DNA was determined by the colorimetric method of Burton (*cf.* Ref. 9). Gradients were calibrated by sonicated calf thymus DNA (~5 S) and ^{3}H-labeled λ(C_{I857} sus-) DNA (Miles Laboratories), and calculations were made by Studier's equation (24) yielding 40 S for λ DNA.

FIG. 2 (*right*). **Alkaline sucrose gradient profile of DNA isolated from whole cardiac ventricles of 42-day-old male Wistar rats.** Two grams of ventricles were minced and rinsed exhaustively with 0.15 M NaCl and 0.1 M EDTA (pH 8.0 at 4 °C), then homogenized (Polytron, setting 4) for 10 s in 10 volumes (w/v) of 0.15 M NaCl and 0.1 M EDTA, pH 8.0, followed by the addition of Na dodecyl sulfate to a final concentration of 2% (w/v). This mixture was agitated for 60 min at room temperature, followed by the isolation of DNA (18) and ultracentrifugal density gradient analysis of DNA as described in the legend to Fig. 1*B*.

2.) The biochemical basis of induced cardiac hypertrophy that is caused by stress-induced overproduction of developmental hormones, is shown in the mechanism of control of RNA synthesis by poly ADP-ribosylation (ref. 6). The molecular basis of this phenomenon is interpreted to be a control of DNA template availability to RNA polymerases, through poly ADP-ribosylation of DNA-binding non histone proteins.

Figure 3. (cf. ref. 6)

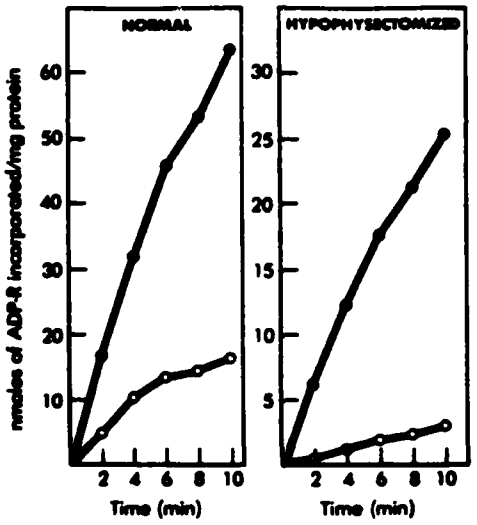
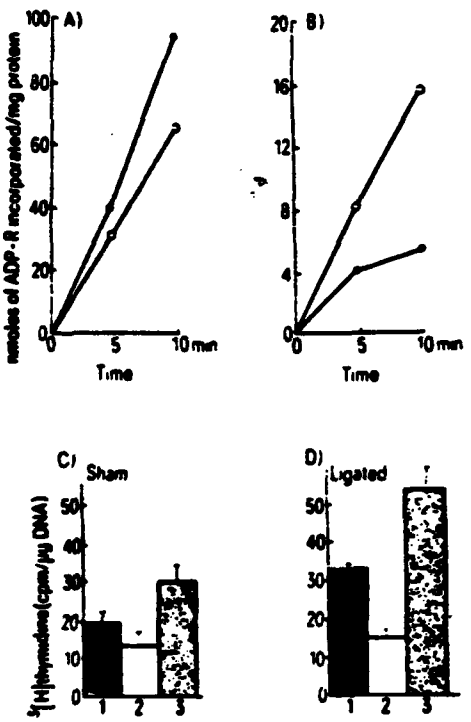


FIGURE 1. Effect of hypophysectomy and L-triiodothyronine treatment of normal and hypophysectomized rats on cardiocyte nuclear polyadenosinediphosphoribose polymerase activity. Polyadenosinediphosphoribose activity in the isolated nuclei was monitored by measuring the rate of incorporation of [^{14}C]NAD into an acid insoluble product as described in detail earlier (12). The reaction mixture at 25 °C in a volume of 235 µl contained 100 mM Tris-HCl, pH 8.2, 2 mM DTT, 20 mM $MgCl_2$, 0.1 mM phenyl methene sulfony; fluoride, 0.5 mM EDTA, 0.5 mM NAD, 2.8×10^7 dpm of [^{14}C]NAD, 80 µg of nuclear protein. (●) Saline treated; (○) L-triiodothyronine treated.

3.) The above mechanism is illustrated in animal experiments, where cardiac hypertrophy was induced by aortic co-arctation (ref.7), and selective- apparently stress induced hormone mediated- responses in poly ADP-ribosylation in cardiocyte nuclei were observed.

Figure 4. (cf ref. 7)

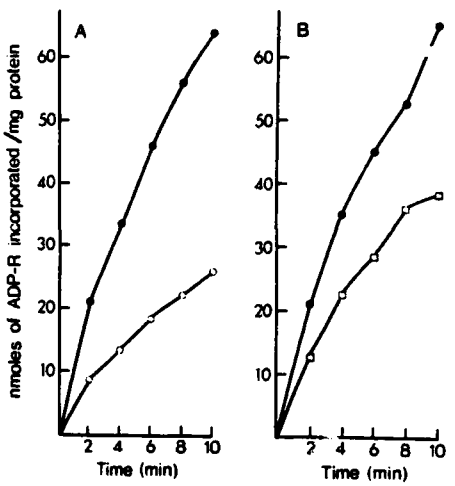


A Poly ADP-R synthetase activity of cardiocyte nuclei. -●-●-, control: -○-○-, after aortic coarctation. *B* Poly ADP-R synthetase activity of non-cardiocyte nuclei. -●-●-, control: -○-○-, after aortic coarctation. *C* [^{3}H] methyl thymidine incorporation into cardiac nuclei. 1. in homogenates; 2. in cardiocyte nuclei; 3. non-cardiocyte nuclei of control (sham operated) animals. *D* [^{3}H] methyl thymidine incorporation into DNA of cardiac nuclei after aortic coarctation. 1. in homogenates; 2. in cardiocytes; 3. in non-cardiocyte nuclei.

The accuracy of the poly ADP-R synthetase assay[9] is within ± 5% (fig. A and B) and the SD of in vivo analyses of thymidine incorporation is shown in figure C and D by error bars.

4.) An age dependent inhibitory effect of steroid hormones on cardiocyte poly ADP-ribosylation has been discovered (ref.8), clearly indicating that in a selected cell type coincidental with age-dependent cessation of DNA synthesis, steroid hormones exert their physiological effect by way of inhibition of poly ADP-ribosylation of non histone proteins. These steroid hormones also stimulate the catabolism of the polymer, a specific effect of steroids, that facilitates de-poly ADP-ribosylation of non histone proteins, thus modifying their probable binding (sites?) on the genome.

Figure 5. (cf. ref. 8)

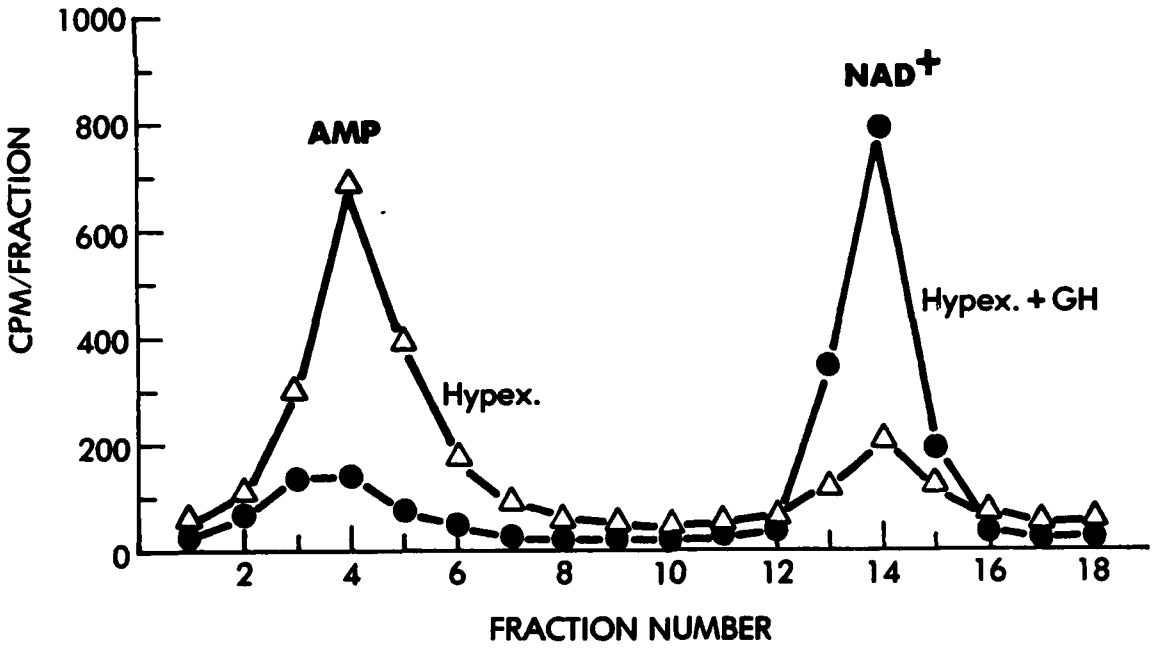


LEGEND TO FIGURE. Rates of poly ADP-R synthesis in vitro by cardiocyte nuclei isolated from 10 normal (-●-●-), 10 hydrocortisone treated (A, -O-O-) and 10 aldosterone treated (B, -□-□-) rats. Assay conditions were described in ref. 1. Parallel determinations did not deviate more than 10% from the mean.

5.) In contrast to the precancerous state where poly ADP-ribosylation is increased (cf. ref. 1,2), growth-hormone induced physiological growth is accompanied by a marked decrease in poly ADP-ribosylation (ref. 9). There is a metabolic crossover (measured by in vivo ^{14}C-ribose-labelling kinetics) in the growth hormone induced growth at the NAD site (whereas in the precancerous state, the metabolic crossover takes place at the polymer itself) as shown in figure 6.

Figure 6.

In vivo metabolic crossover of ribose labelling indicating an inverse labelling of AMP and $^{+}$NAD.



Legend: HPLC-radiochemical isolation of AMP (-Δ-Δ-) and NAD (-●-●-) from hypophysectonized and growth hormone treated rats (liver).

List of Papers that appeared in print during the period of October 1,1981 to September 31, 1982:

1. Romaschin,A.D., Kirsten,E., Jackowski,G., and Kun,E. "Quantitative Isolation of Oligo- and Polyadenosine-diphosphorylated Proteins by Affinity Chromatography from Livers of Normal and Dimethylnitrosamine-treated Syrian Hamsters: In Vivo and in Vitro Metabolism of the Homopolymer." J. Biol. Chem. 256, pp. 7800-7805, 1981.

2. Kun,E., Romascin,A.D., Blaisdell,R.J., and Jackowski,G. "ADP-ribosylation of Nonhistone Chromatin Proteins in Vivo amd of Actin in Vitro and Effects of Normal and Adnormal Growth Conditions and Organ-specific Hormonal Influences," from Metabolic Interconversion of Enzymes 1980, ed. Holzer,H., Springer-Verlag, Berlin, pp. 280-293, 1981.

3. Kirsten,E., Minaga,T., and Kun,E. "Coincidence of Subnuclear Distribution of Poly (ADP-ribose) Synthetase and DNA Polymerase β in Nuclei of Normal and Regenerating Liver." FEBS Letters, 139, pp.117-120, 1982.

4. Kun,E., Minaga,T., Kirsten,E., Jackowski,G., Peller,L., Marton,L., Oredsson,S.M., and Milo,G. "Regulation of Chromatin Fucntion by Polyadenosine Diphosphoribosylation." XIIth Steenbock-Lilly Symposium, June 7-11, Madison, WI, 1982

5. Jackowski,G. and Kun,E. "Age-dependent Variation of Rates of Polyadenosine-diphosphoribose Synthesis by Cardiocyte Nuclei and the Lack of Correlation of Enzymatic Activity with Macromolecular Size Distribution of DNA." J. Biol. Chem., 256, pp.3667-3670, 1981.

6. Jackowski,G. and Kun,E. "The Influence of Triiodothyronine on Polyadenosine-diphosphoribose Polymerase and RNA Synthesis in Cardiocyte Nuclei." J. Mol. Cell. Cardiol.14, pp. 65-70, 1982.

7. Jackowski,G., Heymann,M.A., Rudolph,A.M., and Kun,E. "Cell Specific Response of Cardiac Poly ADP-R and DNA Synthesis to Circulatory Stress." Experientia, 38, pp.1068-1069, 1982.

8. Jackowski,G., Romaschin, A.D., and Kun,E. "Age Dependent Selective Effects of Hydrocortisone and Aldosterone on the Polyadenosine Diphosphoribose Metabolism of Isolated Cardiocyte Nuclei." Biochem. Internat. 4, pp. 17-24, 1982.

9. Romaschin,A.D. and Kun,E. "Decrease of Hepatic Mono and Oligo Adenosine Diphosphoribose Content and Augmentation of [^{14}C]Ribose Incorporation During Induction of Growth by Bovine in Hydrophysectomized Rats." Biochem. Biophys. Res. Commun., 102, pp. 952-957, 1981.

The principal investigator was invited speaker to international symposia and invited lecturer to scientific organizations as follows:

1. International Symposium of Covalent Protein Modification, Firenze, October 1982.

2. Steenbock-Lilly Symposium, University of Wisconsin, June 1982.

3. Plenary Lecture, University of Southern Alabama, May 1982

4. USA-USSR Scientific Conference, Hershey, PA, invited lecturer.

5. Seminar Speaker, Columbia University, Department of Biochemistry, September 1982.

6. Seminar, Institute of Investigaciones Cytologicas, Valencia, Spain, October 1981.

7. Seminar Speaker, Eli Lilly Co.,Indianapolis, July 1982.

8. Seminar Speaker, Bristol Meyer Co., New York, 1982.

Consultant Collaboration:

Ohio State University, Dr. G. Milo, AFOSR 47-620-C-0110

Scientific discussion with Dr. Lewis Thomas, Chancellor, Sloan Kettering Cancer Center, New York

Patent:

Patent concerned with biochemical prevention of carcinogenesis, filed June 1982 through the University of California Patent Office, Berkeley, CA 94720

Personnel:

E. Kun, Principal Investigator

Academic Personnel:

E. Kirsten
A.D. Romaschin, returned to University of Toronto 1981
G. Jackowski
T. Minaga
J. McLick, part-time collaborator without salary
R. Schoenborn, administrative assistant replaced by
C. Paulsen October 1, 1982

Summary:

Work between 1981-1982 provided:

a.) the experimental basis for the biochemical recognition of a critical chromatin response to environmental toxicants, an effect that leads to cancer;

b.) the molecular pharmacological basis of prevention of this fatal toxic effect (of poisons, ionizing radiation, etc.) has been experimentally established; and,

c.) nuclear poly ADP-ribosylation was identified as a physiological control process in hypertrophy, hyperplasia, and hormone action.