The EMBO Journal vol.4 no.6 pp.1491 – 1494, 1985

Tumor promoter phorbol-12-myristate-13-acetate induces poly(ADP)-ribosylation in fibroblasts

Neeta Singh, Guy Poirier and Peter Cerutti

Department of Carcinogenesis, Swiss Institute for Experimental Cancer Research, 1066 Epalinges/Lausanne, Switzerland

Communicated by P.Cerutti

The tumor promoter phorbol-12-myristate-13-acetate (PMA) causes an increase in poly(ADP)-ribosylation in mouse and human fibroblasts *via* the intermediate formation of active oxygen. In contrast to poly(ADP)-ribosylation induced by the methylating agent N-methyl-N'-nitro-N-nitrosoguanidine, *de novo* RNA and protein synthesis are required and the accumulation of the polymer occurs in the absence of detectable DNA strand breakage. Our results suggest a mechanism for PMA-induced modulation of chromatin structure and gene expression.

Key words: tumor promotion/poly(ADP)-ribosylation/active oxygen

Introduction

Tumor promoters modulate the expression of growth- and differentiation-related genes. Many promoters induce a cellular prooxidant state, i.e., they increase the concentration of active oxygen species (hydroxyl-, superoxide-, hydroperoxy-radicals, singlet oxygen, hydrogen peroxide) (Cerutti, 1985). Therefore, the question arises how active oxygen might affect gene expression. Poly(ADP)-ribosylation (ADPR) of chromosomal proteins may represent a mechanism of regulation of gene expression by active oxygen because it is intimately related to the redox state of the cell, DNA strand breakage and chromatin conformation (Hollenberg and Ghani, 1982; Uchigata *et al.*, 1982; Purnell *et al.*, 1980; Mandel *et al.*, 1982; Poirier *et al.*, 1985). Evidence for a role of ADPR in DNA repair (Jacobson *et al.*, 1983; Wielckens *et al.*, 1983; Durkacz *et al.*, 1980), cell differentiation (Althaus *et al.*, 1982; Johnstone and Williams, 1982; Farzaneh *et al.*, 1982) and malignant transformation (Kun *et al.*, 1983; Borek *et al.*, 1984) has been obtained. We now report that the potent mouse skin promoter phorbol-12-myristate-13-acetate (PMA), which produces a prooxidant state in several cell types (see Cerutti, 1985), increased poly(ADPR) levels *via* the intermediacy of active oxygen in mouse embryo fibroblasts C3H10T1/2 and human fibroblasts 3229. In contrast to simple alkylating agents, maximal poly(ADPR) concentrations following PMA treatment are reached only after a delay of 2 – 3 h. Accumulation of poly(ADPR) occurs in the absence of detectable levels of DNA strand breaks, is prevented by anti-oxidants, requires *de novo* protein and RNA synthesis and is not accompanied by a decrease in cellular NAD. We have shown previously that PMA induces a very rapid increase in poly(ADPR) concentration *via* the intermediacy of active oxygen in human blood monocytes. Unlike fibroblasts these cells react to PMA treatment with a massive oxidative burst and extensive DNA breakage (Singh *et al.*, 1985a).

Results and Discussion

Rapidly growing monolayer cultures of human fibroblasts 3229 or mouse embryo fibroblasts C3H10T1/2 were treated with 25 ng/ml PMA, and the cellular concentration of poly(ADPR) was determined by the fluorescence method of Jacobson *et al.* (1984). Figure 1 shows that maximal poly(ADPR) levels were reached in 3 h in 10T1/2 and 2 h in 3229 cells. The increase in concentration was ~10-fold. Poly(ADPR) levels in 10T1/2 cells did not return completely to control values even after 14 h incubation. For comparison, 10T1/2 cells were also treated with the methylating agent N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) which is known to induce poly(ADPR) synthesis with high efficiency (Salinas *et al.*, 1979). Stimulation of poly(ADPR) by 5 or 20 μg/ml MNNG was 7- and 33-fold, respectively, and the maximum was reached already in 20 min. NAD represents the substrate for poly(ADPR) synthesis. Figure 2 shows that the stimulation of poly(ADPR) by 5 μg/ml MNNG resulted in a 35% drop in cellular NAD within 45 min in general agreement with results reported previously (Jacobson *et al.*, 1980). In contrast, NAD concentrations remained essentially constant between 140 and 150 nmol/10^8 cells from 20 min up to 5 h incubation with PMA.

The results of experiments with inhibitors of macromolecular synthesis are summarized in Table I for 10T1/2 cells. Analogous results were obtained for human fibroblasts (Singh *et al.*, 1985b). It is concluded that *de novo* RNA and protein synthesis are necessary for the increase in poly(ADPR) levels caused by PMA. With the exception of the slight inhibition exerted by α-amanitin ($p < 0.200$) the same drugs did not affect the stimulation of poly(ADPR) by MNNG (note: actinomycin D at the concentration used did not inhibit ADP-ribosyl transferase in permeabilized 10T1/2 cells).

To obtain a measure of the total ADP-ribosyl transferase activity present before and after PMA stimulation, 10T1/2 cells were permeabilized (Jacobson *et al.*, 1980) and treated with DNase I (100 μg/ml) according to Berger *et al.* (1978). This treatment induces a large number of DNA breaks and results in maximal stimulation of ADP-ribosyl transferase (Berger *et al.*, 1978; Benjamin and Gill, 1980). From the observation that poly(ADPR) levels following DNase I treatment had increased 2-fold after 3 h of PMA treatment, we conclude that ADP-ribosyl transferase has been produced *de novo* or becomes more responsive to DNA breaks caused by DNase I.

Inhibition of degradation instead of increased rates of synthesis could result in the accumulation of poly(ADPR). To estimate polymer half-life time ($t_{1/2}$), 10T1/2 cells were exposed to PMA for 180 min before the synthesis was inhibited by the addition of 5 mM 3-aminobenzamide. Polymer levels decreased to a half-maximal value within 2 min in agreement with values reported in the literature for alkylating agents (Wielckens *et al.*, 1982). It is evident that PMA did not drastically increase polymer stability in 10T1/2 cells. However, shorter $t_{1/2}$ values cannot readily be determined by this method because the rate of cellular penetra-

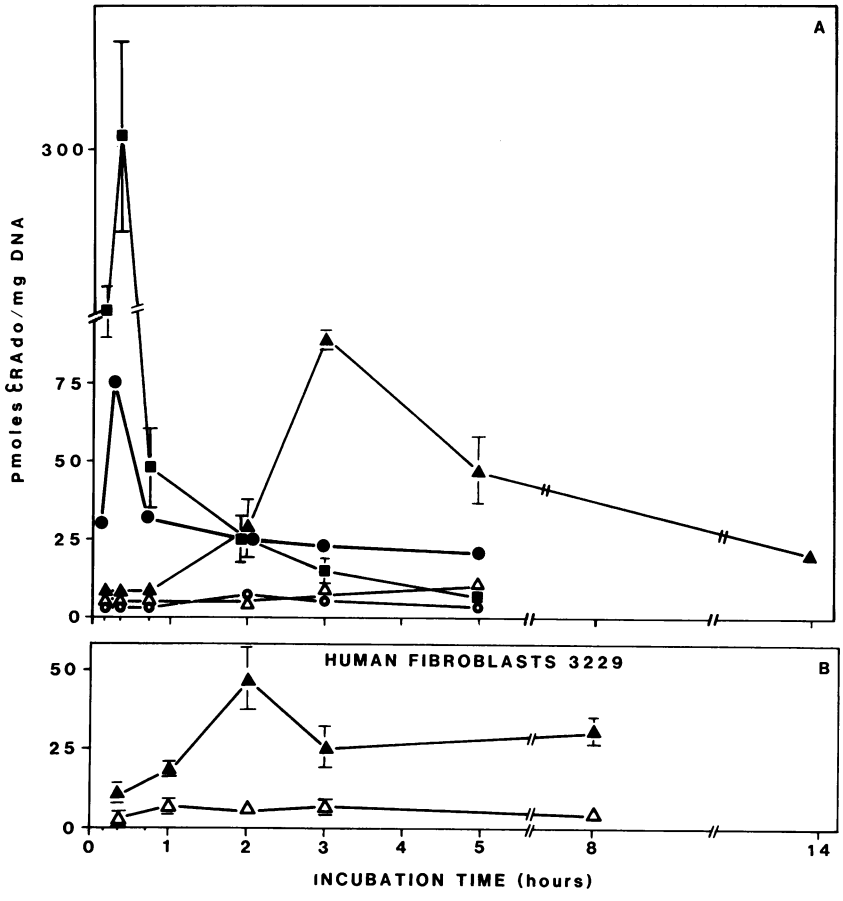

Fig. 1. Kinetics of poly(ADPR) accumulation in fibroblasts treated with PMA or MNNG. Intracellular content of poly(ADPR) following treatment of fibroblasts wth PMA (25 ng/ml) and MNNG (136 μM). Each value is a mean of two separate experiments, each performed in duplicate. (**A**) Mouse embryo fibroblasts C3H 10T1/2. (**B**) Human fibroblasts 3229. (△) Control; (▲) 25 ng/ml PMA; (○) control; (■) 20 μg/ml MNNG; (●) 5 μg/ml MNNG.

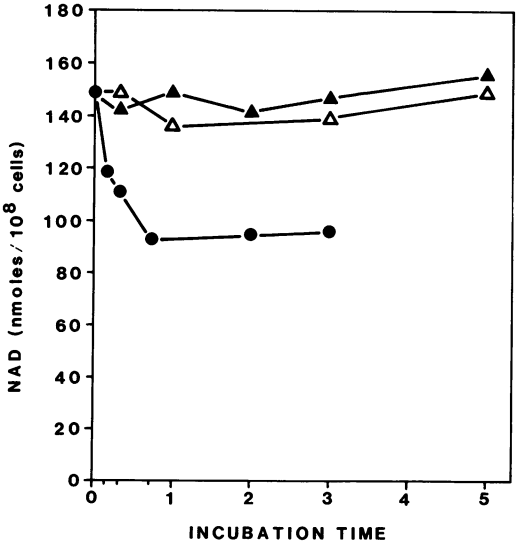

Fig. 2. Effect of PMA and MNNG on NAD concentrations in C3H10T1/2 cells. Concentrations of PMA (25 ng/ml) and MNNG (5 μg/ml) were used which cause a comparable increase in the poly(ADPR) levels (see Figure 1). The NAD concentrations were determined according to Jacobson and Jacobson (1979). (△) control; (▲) 25 ng/ml PMA; (●) 5 μg/ml MNNG.

tion of 3-aminobenzamide becomes limiting. Therefore, it is conceivable that $t_{1/2}$ increased from a lower value to 2 min upon PMA treatment.

Table I. Effects of inhibitors of RNA and protein synthesis on poly(ADPR) levels in C3H 10T1/2 cells

Inhibitors	PMA	MNNG	Poly(ADPR)	% inhibition
–	–	–	9.2 ± 5.0	–
–	+	–	79.0 ± 3.9	0
Actinomycin D	+	–	40.8 ± 4.3	55
α-Amanitin	+	–	26.2 ± 0.2	75
Cycloheximide	+	–	14.6 ± 3.5	100
–	–	–	9.5 ± 3.1	–
–	–	+	315.2 ± 31.7	0
Actinomycin D	–	+	281.1 ± 51.7	11
α-Amanitin	–	+	258.5 ± 21.0	18
Cycloheximide	–	+	321.5 ± 56.5	0

Mouse C3H 1051/2 cells were incubated with PMA (25 ng/ml) for 3 h or MNNG (20 μg/ml) for 20 min and poly(ADPR) levels (pmol εRAdo/mg DNA) were evaluated as described in Materials and methods. Cycloheximide (5 μg/ml), α-amanitin (1 μg/ml), and actinomycin D (2 μg/ml) were added 30 min prior to the treatment with MNNG or PMA. Mean values with standard deviations of two experiments carried out in duplicate are given.

Anti-oxidants should exert an inhibitory effect if the induction of a cellular prooxidant state is necessary for the accumulation of poly(ADPR) caused by PMA. Experiments with enzymes which destroy active oxygen species, i.e, CuZn-superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-P), and the diffusable anti-oxidant butylated-hydroxytoluene (BHT) are summarized in Table II. It is evident that all these

Table II. Effect of anti-oxidants on the accumulation of poly(ADPR) in PMA and MNNG-treated C3H 10T1/2 cells

Anti-oxidants	PMA	MNNG	Poly(ADPR)	% inhibition
–	–	–	9.2 ± 5.0	–
–	+	–	79.0 ± 3.9	0
SOD	+	–	16.0 ± 4.8	90
CAT	+	–	20.7 ± 5.4	83
GSH-P	+	–	17.0 ± 2.2	89
BHT	+	–	25.7 ± 11.9	76
Heated CAT	+	–	82.0 ± 9.8	0
Heated GSH-P	+	–	55.1 ± 4.1	34
BSA	+	–	75.9 ± 12.5	4
–	–	–	9.5 ± 3.1	–
–	–	+	315.2 ± 31.7	0
SOD	–	+	287.9 ± 55.0	9
CAT	–	+	315.6 ± 66.3	0
GSH-P	–	+	273.4 ± 66.2	13

Mouse C3H 10T1/2 cells were pre-incubated for 30 min with the above antioxidants SOD (25 μg/ml), CAT (50 μg/ml), GSH-P (0.3 μU/ml) or BHT (10 μM) prior to the addition of PMA (25 ng/ml) or MNNG (20 μg/ml). Poly(ADPR) levels (εRAdo pmol/mg DNA) were measured as described in Materials and methods after 3 h of PMA treatment and 20 min for MNNG treatment. Mean values with standard deviations of two experiments carried out in duplicate are given.

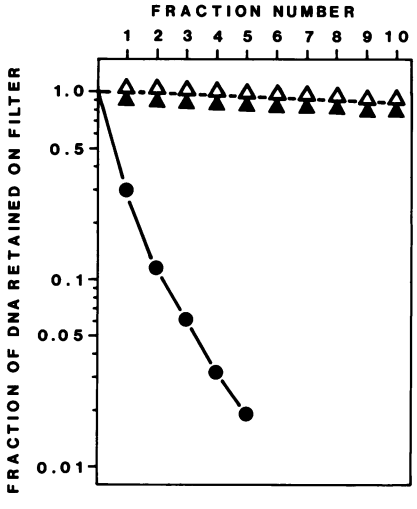

Fig. 3. Alkaline elution of DNA from PMA- and MNNG-treated C3H10T1/2 cells. Concentrations of PMA (25 ng/ml) and MNNG (5 μg/ml) were used which cause a comparable increase in poly(ADPR) levels (see Figure 1). Alkaline elution was carried out according to Kohn *et al.* (1976). (△), untreated control; (▲), 25 ng/ml PMA; (●), 5 μg/ml MNNG.

anti-oxidants are potent inhibitors for the accumulation of poly(ADPR) by PMA but not by MNNG. Heated CAT (boiling for 5 min at pH 7.4) and GSH-P (heated for 2 h at 60°C at pH 7.2 resulting in ~70% inactivation of the enzyme) had lost most of their inhibitory capacity (note: heating of SOD does not readily inactivate the enzyme). Bovine serum albumin showed no protection. We conclude that active oxygen represents an intermediate in the accumulation of poly(ADPR)-induced by PMA. The fact that the anti-oxidant enzymes exerted their effect upon extracellular addition suggests that they act at the cell membranes or in the culture medium but their metal co-factors may be transported to the interior of the cell. In contrast, the induction of poly(ADPR) synthesis by MNNG was not affected by the same anti-oxidants.

For cells treated with alkylating agents, ionizing radiation and u.v. light the formation of DNA strand breaks by the damaging agent or as a consequence of repair processes appears to be a prerequisite for the stimulation of poly(ADPR) synthesis. In analogy to ionizing radiation which causes DNA breaks by OH radicals, active oxygen induced by PMA may introduce DNA breakage. This is readily demonstrated for phagocytic leukocytes (Birnboim, 1982), which produce a massive oxidative burst, but is difficult to ascertain for other cell types. The alkaline elution method (Kohn *et al.*, 1976) and the alkaline unwinding assay (Birnboim, 1982) were used to monitor DNA strand breakage. Concentrations of the two agents were chosen which result in the accumulation of comparable amounts of poly(ADPR) (see Figure 1). As shown in Figure 3 for the former method, 25 ng/ml PMA did not cause any measurable breakage. In contrast, 5 μg/ml MNNG induced extensive DNA breakage. Analogous results were obtained by the alkaline unwinding assay (not shown). The induction of a prooxidant state by PMA requires active cellular metabolism and experiments have to be carried out at 37°C, preferably in complete culture medium. Because DNA breaks are very rapidly re-sealed under these conditions we cannot expect large numbers of steady-state breaks as a consequence of PMA treatment. However, even if a small number of (undetectable) breaks were induced by PMA they may not result in poly(ADPR) accumulation to the observed degree.

The major characteristics of poly(ADPR) accumulation induced by PMA are: (i) maximal poly(ADPR) levels are reached only after 2–3 h in contrast to MNNG for which a maximum is attained in 20 min; (ii) the PMA-induced increase in poly(ADPR) levels does not result in a decrease in the cellular NAD concentration; (iii) *de novo* protein and RNA synthesis are required and total poly(ADP)-ribosyl transferase activity increases only slightly; (iv) antioxidants prevent the accumulation of poly(ADPR); (v) there is no convincing evidence for the formation of DNA breaks by PMA in fibroblasts. These characteristics clearly distinguish the mechanisms leading to the increase in poly(ADPR) levels by PMA and by the alkylating agent MNNG. They are reminiscent of poly(ADPR) accumulation in starved Ehrlich ascites cells (Wielckens *et al.*, 1983) heat-shocked (Duran-Torres *et al.*, 1984) or picolinic acid-treated mouse cells, and glycerol-treated HeLa cells (Kidwell and Purnell, 1983). In none of these cases, which can be considered as different facets of cellular stress, is there clear evidence that DNA breaks were required for the augmentation of poly(ADPR) concentrations. However, in view of the strong evidence from the literature that DNA containing breaks represents an obligatory co-factor for the stimulation of ADP-ribosyl transferase, it cannot be excluded that accumulation of poly(ADPR) under these conditions is caused by a very small number of breaks which are not detectable with present technologies. If this is so the 2-fold enhancement in ADP-ribosyl transferase activity in PMA-treated 10T1/2 cells could suffice to account for the ~10-fold increase in poly (ADPR) levels. Increased transferase activity may be an early sign of the mitogenic effect of PMA as is the case for phytohemaglutinin stimulated lymphocytes (Johnstone and Williams, 1982; Greer and Kaplan, 1983). The following alternative mechanisms should be considered. (i) PMA treatment includes an inhibitor of poly (ADPR) breakdown which does not measurably increase polymer half-life time within the limits of present methodology. (ii) PMA induces conformational changes in DNA and chromatin of fibroblasts (but no *bona fide* breaks) which result in the stimulation of poly(ADPR)-transferase. Preliminary results indicate that

several chromosomal proteins are poly(ADP)-ribosylated in PMA-treated 10T1/2 cells (Singh and Cerutti, unpublished. Our results suggest a mechanism for PMA-induced modulation of chromatin structure and gene expression.

Materials and methods

Cell culture

Monolayer cultures of C3H10T1/2 mouse embryo fibroblasts were grown at 37°C in Eagle's basal medium supplemented with 10% heat-inactivated fetal calf serum. Human fibroblasts obtained from newborn foreskin were grown under similar conditions except that Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum was used.

Cell treatment

The cells were treated in complete medium containing serum with PMA (25ng/ml) from a stock solution in acetone or MNNG (5 or 20 μg/ml), in dimethyl sulphoxide, respectively. Controls received an equal amount of acetone (final concentration 0.025%) or dimethyl sulphoxide (final concentration 0.015%). At the indicated times of treatment the cultures were chilled, the medium removed and the cells precipitated with 20% trichloroacetic acid (TCA). Acid-insoluble material was scraped from the dish with a rubber policeman, transferred to a centrifuge tube and collected by centrifugation at 800 *g* for 10 min. The pellet was washed twice with ice-cold 20% TCA and twice with diethyl ether, dried and stored at −20°C until further analysis.

Poly(ADP-ribose) estimation

Polymer levels were determined by the method of Jacobson *et al.* (1984). The poly(ADP)-ribose contained in the acid-insoluble material was purified by dihydroxy-boryl-Sepharose affinity chromatography and digested to nucleosides with snake venom phosphodiesterase and bacterial alkaline phosphatase. The nucleosides were treated with chloroacetaldehyde to form the fluorescent derivative 1,N^6-ethenoribosyl adenosine (εRAdo). εRAdo was separated by h.p.l.c. and quantified by fluorescence determination. Recovery controls were routinely performed by running ^{3}H-labelled poly(ADPR) through the identical protocol. Poly(ADPR) concentrations are expressed as pmol εRAdo/mg DNA. The method of Fiszer-Szafarz *et al.* (1981) was used for DNA estimation.

Acknowledgements

We thank Dr. T.Ochi for carrying out the alkaline elution analysis for DNA strand breakage, Dr. M.K.Jacobson for valuable advice, Ms I.Zbinden for excellent technical assistance, the Swiss National Science Foundation and the Swiss Association of Cigarette Manufacturers for research support, the International Union against Cancer for an Eleanor Roosevelt fellowship to one of us (G.P.) and a post-doctoral fellowship by the Swiss Government (N.S.).

References

Althaus,F.R., Lawrence,S.D., He,Y.Z., Sattler,G.L., Tsukada,Y. and Pitot,H.C. (1982) *Nature*, **300**, 366-368.

Benjamin,R.C. and Gill,D.M. (1980) *J. Biol. Chem.*, **255**, 10502-10508.

Berger,N.A., Weber,G., Kaichi,A.S. and Petzold,S.J. (1978) *Biochim. Biophys. Acta*, **509**, 105-117.

Birnboim,H. (1982) *Science (Wash.)*, **215**, 1247-1249.

Borek,C., Morgan,W.F., Ong,A. and Cleaver,J.E. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 243-247.

Cerutti,P. (1985) *Science (Wash.)*, **227**, 375-381.

Duran-Torres,G.,Juarez-Salinas,H. and Jacobson,M.K. (1984) *Fed. Proc.*, **43**, 2064.

Durkacz,B., Omidiji,O., Gray,D. and Shall,S. (1980) *Nature*, **283**, 593-596.

Farzaneh,F., Zalin,R., Brill,D. and Shall,S. (1982) *Nature*, **300**, 362-366.

Fiszer-Szafarz,B., Szafar,D. and Guevara de Murillo,A. (1981) *Biochemistry (Wash.)*, **110**, 165-170.

Greer,W.L. and Kaplan,J.G. (1983) *Biochem. Biophys. Res. Commun.*, **115**, 834-840.

Hollenberg,M. and Ghani,Q.P. (1982) in Hayaishi,O. and Ueda,K. (eds.), *ADP Ribosylation Reactions: Biology and Medicine*, Academic Press, NY, pp. 439-450.

Jacobson,E.L. and Jacobson,M.K. (1979) *J. Cell. Physiol.*, **99**, 417-426.

Jacobson,E.L., Antol,K.M., Juarez-Salinas,H. and Jacobson,M.K. (1983) *J. Biol. Chem.*, **258**, 103-107.

Jacobson,M.K., Levi,V., Jaurez-Salinas,H., Barton,R.A. and Jacobson,E.L. (1980) *Cancer Res.*, **40**, 1797-1802.

Jacobson,M.K., Payne,D.M., Juarez-Salinas,H., Sims,J.L. and Jacobson,E.L. (1984) *Methods Enzymol.*, **106**, 483-494.

Johnstone,A.P. and Williams,G.T. (1982) *Nature*, **300**, 368-370.

Kidwell,W.R. and Purnell,M.R. (1983) in Miwa,M., Hayaishi,O., Shall,S., Smulson,M. and Sugimura,T. (eds.), *ADP-Ribosylation, DNA Repair and Cancer*, Scientific Societies Press, Tokyo, pp. 243-252.

Kohn,K., Erickson,L., Ewig,R. and Friedman,C. (1976) *Biochemistry (Wash.)*, **15**, 4629-4637.

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

Mandel,P., Okazaki,H. and Niedergang,C. (1982) *Prog. Nucleic Acids Res. Mol. Biol.*, **27**, 1-51.

Poirier,G.G., Brown,T.C. and Cerutti,P.A. (1985) *Carcinogenesis*, **6**, 283-287.

Purnell,M.R., Stone,P.R. and Whish,W.J.D. (1980) *Biochem. Soc. Trans.*, **8**, 215-227.

Salinas,J.H., Sims,J.L. and Jacobson,M.K. (1979) *Nature*, **282**, 740-741.

Singh,N., Poirer,G. and Cerutti,P.A. (1985a) *Biochem. Biophys. Res. Commun.*, **126**, 1208-1214.

Singh,N., Poirier,G. and Cerutti,P.A. (1985b) in *Proceedings of the 7th International Symposium on ADP-Ribosylation Reactions*, Springer Verlag, NY, in press.

Uchigata,Y., Yamamoto,H., Kawamura,A. and Okamoto,H. (1982) *J. Biol. Chem.*, **257**, 6084-6088.

Wielckens,K., Schmidt,K., George,E., Bredehorst,R. and Hilz,H. (1982) *J. Biol. Chem.*, **257**, 12872-12877.

Wielckens,K., George,E., Pless,T. and Hilz,H. (1983) *J. Biol. Chem.*, **258**, 4098-4104.

Received on 28 December 1984; revised on 8 March 1985