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# Enzymatic Toxicogenation of "Activated" Cyclophosphamide by 3′-5′ Exonucleases*

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**Summary.** 3′-5′ Exonucleases from various sources were found to toxicogenate 4-hydroxycyclophosphamide ("activated" cyclophosphamide) by splitting the oxazaphosphorinane ring and releasing an alkylating moiety and acrolein. Neither cyclophosphamide (CP) nor the deactivated metabolites of CP, 4-keto-CP and carboxyphosphamide nor 4-(S-ethanol)-sulfido-CP were attacked by 3′-5′ exonucleases. DNA polymerases with proofreading activity, such as DNA polymerase I from *E. coli* or DNA polymerase $\delta$ from rabbit bone marrow, exhibited a tenfold higher specific activity with "activated" CP than "plain" 3′-5′ phosphodiesterases such as snake venom phosphodiesterase or 3′,5′cyclic AMP phosphodiesterase from bovine heart tissue.

High levels of toxicogenating activity were estimated in peripheric human lymphocytes and tissues of lymphatic origin, suggesting that enzymatic toxicogenation plays a key role with respect to the cytotoxic specificity of "activated" CP.

**Key words:** "Activated" CP – 3′-5′ Exonucleases – Lymphatic tissues – Cytotoxic specificity

## Introduction

The metabolism of CP has been shown to proceed in three major steps: (1) Enzymatic *activation* of CP to 4-hydroxycyclosphosphamide (4-OH-CP) and its tautomer aldophosphamide (both termed as "activated" CP) by mixed-function hydroxylases which occur mainly in the liver (Brock and Hohorst 1962). (2) *Deactivation* of "activated" CP to 4-keto-CP and carboxyphosphamide either by aldehyde dehydrogenases (Hill et al. 1970; Struck et al. 1971) or nonenzymatically by reaction with thiol compounds such as protein-SH groups to yield 4-(SR)-sulfido-CP (Draeger et al. 1976; Peter et al. 1976). (3) Spontaneous *toxicogenation* of aldophosphamide by $\beta$-elimination of acrolein to yield phosphoramide mustard as the probable ultimate alkylating and cytotoxic metabolite of CP.

Recently we found that rat serum and various tissues of rats were able to toxicogenate "activated" CP enzymatically (Voelcker et al. 1981) at much higher rates compared with spontaneous toxicogenation. The data presented below demonstrates that 3′-5′ exonucleases are enzymes which toxicogenate 4-OH-CP.

## Materials and Methods

*Enzymes*

The DNA polymerase $\delta$ was prepared from erythroid hyperplastic rabbit bone marrow according to Byrnes et al. (1976) and purified 480-fold (ammonium sulfate precipitate). All other enzymes were obtained from commercial sources: DNA polymerase I, DNA polymerase I large fragment, exonuclease III (*E. coli*) from Worthington; phosphodiesterase I (snake venom), phosphodiesterase II (calf spleen), nuclease $P_1$ (penicillinum citrium), deoxyribonuclease I (bovine pancreas), 3′,5′ cyclic AMP phosphodiesterase (bovine heart), ribonuclease A (bovine pancreas) from Boehringer/Mannheim, FRG

*Substrates*

4-OH-CP and 4-hydroperoxycyclophosphamide (4-OOH-CP) were prepared by ozonization of CP according to Peter et al. (1979); desoxythymidine triphosphate ([$^3$H] dTTP) was obtained from Amersham Buchler and poly (dA–dT) from Miles Laboratories. [$^3$H]poly (dA–dT) as a substrate for the 3′-5′ exonuclease assay was synthesized using [$^3$H] dTTP according to Byrnes et al. (1977).

*Assays*

All enzyme reactions were carried out at 37 °C. Toxicogenating activity was determined by measuring the acrolein release from 4-OOH-CP or 4-OH-CP. The enzyme preparation to be tested was added (1 mg protein/ml) to a reaction mixture containing 4-OH-CP (1 m*M*) in 0.07 *M* phosphate buffer pH 6. The liberated acrolein was

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**Table 1.** Toxicogenation of 4-hydroperoxycyclophosphamide by various phosphodiesterases

| Enzyme | Specific activity (nmol/min per mg protein) |
|---|---|
| Phosphodiesterase I from snake venom (EC 3.1.4.1) exonuclease 3'–5' | 4.2 |
| Phosphodiesterase II from calf spleen (EC 3.1.16.1) exonuclease 5'–3' | 0.0 |
| Exonuclease III from *E. coli* (EC 3.1.4) specific for double-stranded DNA | 0.0 |
| Nuclease P1 from penicillinum citrium (EC 3.1.4) exonuclease 3'–5', DNA and RNA | 2.3 |
| Deoxyribonuclease I from bovine pancreas (EC 3.1.21.1) endonuclease | 0.0 |
| 3', 5' cyclic AMP phosphodiesterase (EC 3.1.4.17) from bovine heart | 0.7 |
| Ribonuclease I (EC 3.1.27.5) from bovine pancreas | 0.0 |

Specific activity was determined by measuring the acrolein release from 4-OOH-CP (3.4 m*M*) in 0.07 *M* phosphate buffer pH 6 at 37 °C. Protein concentration in the reaction mixture was 1 mg protein/ml. For details see Methods

**Table 2.** Toxicogenation of 4-hydroxycyclophosphamide and 4-hydroperoxycyclophosphamide by DNA polymerase-associated 3'–5' exonucleases

| Enzyme | Specific activity (nmol/min per mg protein) | |
|---|---|---|
| | 4-OOH-CP | 4-OH-CP |
| DNA polymerase $\delta$/3'–5' exonuclease from rabbit bone marrow | 97.0 | No determination |
| DNA polymerase I/3'–5' exonuclease from *E. coli* (EC 2.7.7.7) | 31.3 | 46.7 |
| DNA polymerase I large fragment/ 3'–5' exonuclease from *E. coli* | No determination | 37.8 |

Specific activity was determined by measuring the acrolein release from 4-OOH-CP (3.4 m*M*) or 4-OH-CP (1 m*M*) in 0.07 M phosphate buffer pH 6 at 37 °C. Protein concentration in the reaction mixture was 1 mg protein/ml. DNA polymerase $\delta$ from rabbit bone marrow contained 0.31 units DNA polymerase and 254 units 3'–5' exonuclease per mg protein. For details see Methods

flowed over with $N_2$ and trapped in four ice-cooled washing flasks in sequence, each containing 1.5 ml solution of 1.7 mg/ml aminophenol and 2 mg/ml hydroxylammonium x HCl in 1 *N* HCl. At 10-min intervals the acrolein content in the washing flasks was determined fluorometrically according to Alarcon (1968). Acrolein released from 4-OOH-CP (3.4 m*M*) was measured after 10, 20, 30, and 60 min incubation in 0.07 *M* phosphate buffer pH 6 by HPLC. We used a micro-bondapak $C_{18}$-column (Waters, Massachusetts, USA) and 0.07 *M* phosphate buffer pH 7/methanol (85:15/v:v) as eluant. Acrolein was detected by photometry at 206 nm. The DNA polymerase was tested with poly (dA–dT) as template/primer; 3'-5' exonuclease was assayed by measuring the release of [$^3$H] dTMP from 3' terminally labelled poly (dA–dT). One unit of DNA polymerase $\delta$ catalizes the incorporation of 1 pmol of dTMP/min at 37 °C. The exonuclease activity was expressed as the amount of radioactivity in dpm released from labelled poly (dA–dT)/min at 37 °C.

**Table 3.** Toxicogenation of 4-hydroxycyclophosphamide by human lymphocytes

| Lymphocytes subject | Activity (nmol/min per mg protein) |
|---|---|
| Vo. | 22.0 |
| V. | 67.0 |
| Bi. | 50.0 |
| St. | 34.0 |
| Jo. | 28.0 |
| Human plasma | 0.56 |

Human blood lymphocytes obtained from normal subjects were prepared and purified by the one-step sodium metrizoate/ficoll centrifugal procedure. Activity was determined by measuring the acrolein release from 4-OH-CP (1 m*M*) in phosphate buffer pH 6 at 37 °C with suspended lymphocytes (protein concentration in the reaction mixture approximately 0.1–0.3 mg/ml) or with human blood plasma (protein concentration in the reaction mixture approximately 1 mg/ml)

## Results and Discussion

As shown in Tables 1 and 2, only phosphodiesterases (exonucleases) that are capable of splitting monostranded DNA in the 3'-5' direction or of splitting 3',5'cyclic AMP were able to toxicogenate "activated" CP. CP and its deactivated metabolites, 4-keto-CP and carboxyphosphamide, were not hydrolyzed by these enzymes. Endonucleases, ribonucleases, protein phosphatases, and acid and alkaline phosphatases were found to be inactive.

Strikingly high levels of activity were exhibited by 3'-5' exonucleases linked to DNA polymerases, e.g., DNA polymerase I from *E. coli* (EC 2.7.7.7) or DNA polymerase $\delta$ from rabbit bone marrow (Table 2). It seems that these enzymes, which are directly involved in cell proliferation and show proofreading activity (Brutlag and Kornberg 1972; Byrnes et al. 1976; Lee et al. 1980; Lee et al. 1981), have an unexpected specificity for "activated" CP.

The exonuclease and DNA polymerase activities are within the same protein molecule. Therefore the release of the alkylating moiety from "activated" CP by the action of the exonuclease subunit may lead to a specific alkylation of the DNA polymerase subunit, thus influencing DNA synthesis in the cell. We assume that the molecular basis for the relatively high cancerotoxic selectivity and cytostatic specificity of "activated" CP (Brock and Hohorst 1977; Hohorst et al. 1976) could be explained by this mechanism.

First attempts to estimate the 4-OH-CP toxicogenating activity in various tissues revealed its presence mainly in lymphatic tissues such as spleen and thymus. The highest activities were assayed in unstimulated human lymphocytes (Table 3). No activities were found in liver, muscle, heart, brain or red blood cells of rats. Whether the 4-OH-CP toxicogenating activity measured in lymphocytes, lymphatic tissues, and serum is identical with 3′-5′ exonucleases and particularly with DNA-polymerase-linked 3′-5′ exonucleases remains open to further detailed investigations. However, results of our experiments with adenosine 5′ monophosphate (5′AMP) strongly support such an assumption: 5′AMP, a potent inhibitor of 3′-5′ exonucleases linked to DNA polymerases (Byrnes et al. 1977), also competitively inhibits the toxicogenating activity of rat serum ($K_M = 2 \times 10^{-3}$ mol/l, $K_i = 5 \times 10^{-3}$ mol/l; both measured with 4-OH-CP as substrate).

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