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INVESTIGATIONS ON THE MECHANISM OF DNA EXCISION REPAIR IN TISSUE CULTURE CELLS

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KURZFASSUNG

UNTERSUCHUNGEN DES DNA- EXZISIONSREPARATUR- MECHANISMUS IN ZELLKULTUREN.

Es wurde der Einfluß verschiedener Temperaturen und Hemmstoffe, wie p-Chlormercuribenzoat oder Cytosin-Arabinosid, auf die semikonservative DNA- Synthese und die DNA- Reparatur in HeLa Zellen und Milzzellen untersucht. In Parallelversuchen wurden zwei Enzymvarianten von DNA- Polymerase aus Schweinemilz eingesetzt. Dabei zeigen sich Korrelationen zwischen α- Polymerase und semikonservativer DNA- Synthese bzw. zwischen β- Polymerase und DNA- Reparatur. Diese Übereinstimmung ist unter der Einwirkung verschiedener Temperaturen besonders deutlich.

Deskriptoren: DNA- SYNTHESE / DNA- REPARATUR / DNA- POLYMERASE / HYPERTHERMIE

SUMMARY

INVESTIGATIONS ON THE MECHANISM OF DNA EXCISION REPAIR IN TISSUE CULTURE CELLS.

Semiconservative DNA- synthesis and repair- synthesis was measured in HeLa cells and spleen cells under different conditions (i.e. different temperatures, addition of p-chloromercuribenzoate or cytosine-arabinoside). In order to obtain more information about the enzymatic background of these steps of DNA metabolism, parallel in vitro experiments were done with two different types of DNA polymerase, which had been isolated from pig spleen. At least the experiments at different temperatures are showing some correlations of α-polymerase with semiconservative synthesis and of β-polymerase with repair synthesis.

Descriptors: DNA- SYNTHESIS / DNA- REPAIR / DNA - POLYMERASE / HYPERTHERMIA

INVESTIGATIONS ON THE MECHANISM OF DNA EXCISION REPAIR IN TISSUE CULTURE CELLS

INTRODUCTION

In most of the investigated eukaryotic cells there are two main types of enzymatic activity of DNA dependent DNA- polymerase, recently called α- and β- polymerase (1). Not only mammalian cells, also all vertebrates and most of the lower animals contain both types (2). The properties of the enzymes of one group from different animals or different tissues are very similar, the α- polymerase has the higher molecular weight (about 15o ooo) and is more sensitive to the common inhibitors than the smaller β- polymerase (about 5o ooo) (3). There are evidences that these enzymes play different roles in the cell, so the β- polymerase always remains constant, but the α- polymerase activity is increased in proliferating cells, regenerating tissue and tumors (4, 5, 6, 7). HEADASI and WEISSBACH (8) determined the development of the enzymatic activity during the cell cycle and found that the activity of the α- polymerase increases during the S- phase. This indicates that the α - enzyme is important for semiconservative synthesis and one could postulate that - on the other hand - the β - enzyme is involved in repair processes. In order to test this hypothesis and to find out which enzyme is

responsible for repair we run parallel experiments with both living cells and isolated enzymes under different conditions and measured the uptake of activity (^{3}H-TdR) into the DNA.

METHODS

The polymerases had been isolated and purified from pig spleen as described previously (9) and assayed by the filter precipitation technique (1o). The enzymes were incubated for 15 minutes with 13/ug of activated DNA (nicked by a short treatment with DNase I), with 65 nMol of each of the four necessary deoxynucleotidtriphosphates (the thymidine triphosphate labeled with o,25/uCi ^{3}H-dTTP), 5 mM $MgCl_2$, 1 mM EDTA, 1 mM 2-mercaptoethanol and 5o mM Tris-HCl buffer of the pH optimum for the different enzymes (table 1) in a total volume of 125/ul. The radioactivity of the acid precipitable material was measured.
Semiconservative DNA synthesis was determined by incubation of either spleen cells (in suspension) or HeLa cells (grown on petri dishes) with 2/uCi/ml of ^{3}H-methylthymidine (5o Ci/mMol) for 3o minutes and determination of the specific activity of the DNA.
For the studies of repair synthesis, we preincubated the cells 3o minutes with 1o mM hydroxyurea - this treatment blocks nearly 95% of semiconservative DNA synthesis. The cells were irradiated with 1oo erg/mm^2 UV light of 254 nm, an incubation period of 6o minutes with 2/uCi ^{3}H-TdR/ml followed and finally the specific activity of the DNA was estimated. The amount of DNA was determined by measurement of the extinction (26o nm) directly in an acid hydrolysate.
All incubations of cells were done in Hanks buffer. When inhibitors were applied, they had been added 3o minutes before addition of the labeled precursor.

RESULTS

After disruption of the spleen cells and fractionated centrifugation, we obtained two sources of enzymatic activity, one is the fraction containing most of the nuclei, the other one is the supernatant from 105 000 g - we call it the cytoplasmic fraction. After further purification we separated the material on DEAE- cellulose and received two types of enzymes in both fractions as shown in fig. 1. For the following experiments, we took as representatives for each type of enzymatic activity the first of the two α- peaks from cytoplasm and the β- peak from the nuclear fraction, and purified once more by affinity chromatography on DNA- cellulose.
The characteristic properties of the enzymes are presented in table 1; note that there is no difference between the first and the second part of the α double peak. All of these values correspond to the results of other authors, already published.
Fig. 2 shows the inhibition by p- chloromercuribenzoate. As this substance reacts with all SH- groups in the cell, the inhibition of semiconservative synthesis and repair synthesis could not exclusively depend from its action on the DNA polymerases. A much better and more specific inhibitor is cytosine- arabinoside (fig. 3), it inhibits semiconservative synthesis more than repair synthesis and similar α- polymerase more than β- polymerase.
Fig. 4 shows the influence of different incubation temperatures, note that the β- polymerase, which had been less sensitive to the inhibitors is more sensitive to higher temperatures and so is repair synthesis.
All results presented in the last three figures are done with HeLa cells. Similar experiments were performed with spleen cells, there is less thymidine uptake in all cases because of their lower metabolism, but we found no essential difference between the two cell- systems.

DISCUSSION

In the experiments comparing the influences of an inhibitor in cellular and cell- free systems we can not avoid the inaccuracy of changed local concentrations of that inhibitor, i.e. we can easily adjust the concentration in the cell- free system, but we do not know the actual concentrations in the cell or in the nucleus. This concentration could be higher - if there occur some active transport phenomena - or lower - as a matter of slow penetration or protection by other proteins - than in the medium outside the cells.
Another problem are concurred effects as these substances do not react very specific. The fictitious stimulation of semiconservative synthesis in the case of low concentrations of p-chloromercuribenzoate (fig. 3) is more probably caused by some intoxication of a preforming pathway which leads to a smaller but higher labeled thymidine pool.
Whereas the experiments with p-chloromercuribenzoate give ambigous results, cytosine-arabinoside is much more specific. Assuming a possible active concentration of the inhibitor within the cells, that means each concentration of the cellular experiments corresponds to a higher actual concentration, there is a good correlation of semiconservative DNA synthesis with α- polymerase and repair synthesis with β- polymerase.
Temperature is one parameter, which is independent from cellular surrounding and the temperature- dependances of the reactions in consideration show not only a good correlation of the corresponding maxima - the maximum of the enzyme activity is always 2° higher than that of the corresponding cellular effect - also the shape of the curves is very similar. This would indicate that the polymerases are really the step which determines the speed of the processes.

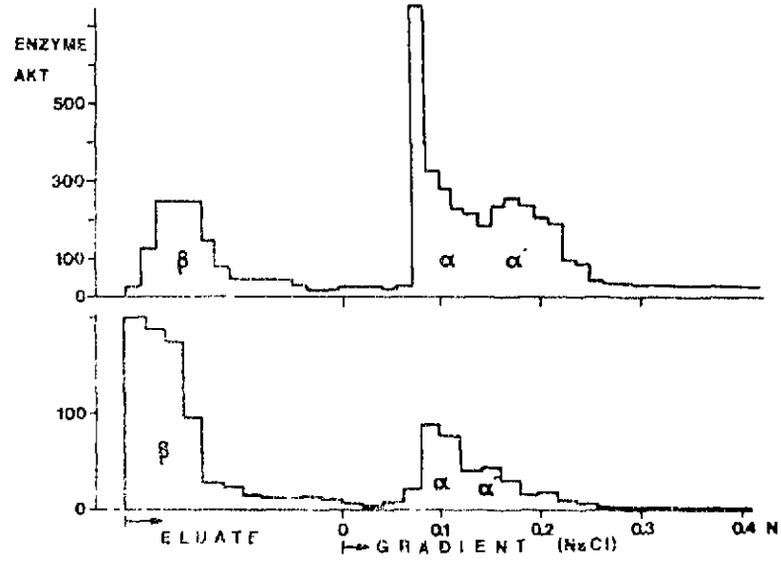
TABLE 1 :

Enzyme	α (cytopl.)	α' (cytopl.)	β (nuclear)
Sedimentation constant	7,3s	7,3s	3,9s
pH- optimum	8,1	8,1	9,5
Mg^{++}- optimum	4.10^{-3}M	4.10^{-3}M	6.10^{-3}M
Inhibition by NaCl:			
0,05 M	26%	24%	95%
0,1 M	6%	3%	91%
0,2 M	< 1%	< 1%	57%

(% activity of control)

Characteristic properties of DNA polymerases from pig spleen.

Fig. 1 :

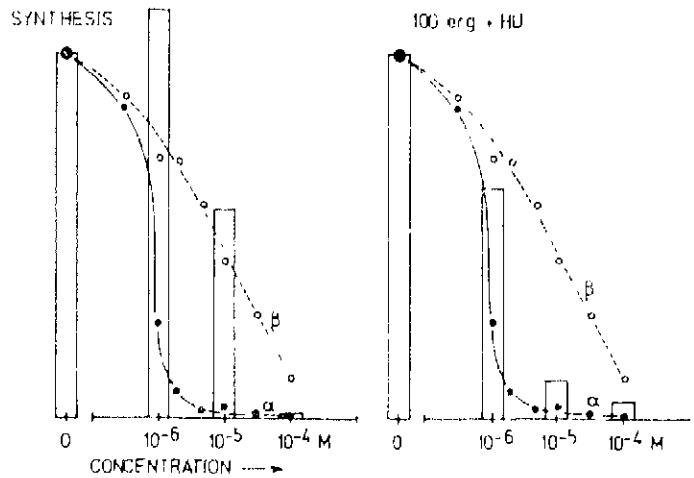


DNA polymerases in extracts of spleen cells, separation on DEAE cellulose.

above: cytoplasmic fraction
below: nuclear fraction

Fig. 2 :

p- CMB

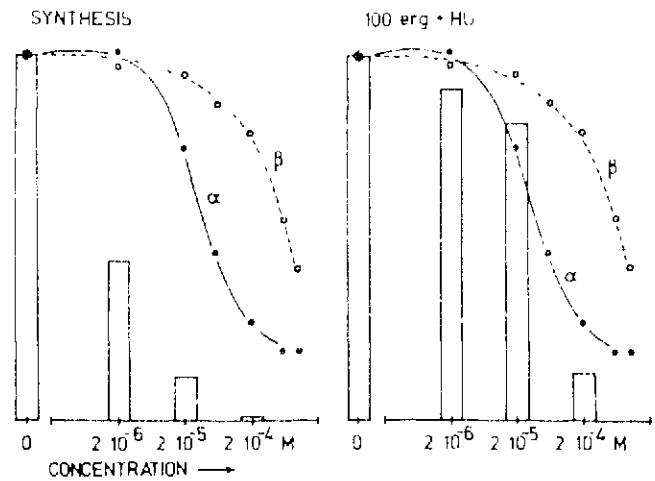


Inhibition by p-chloromercuribenzoate.

All values in relation to an untreated control (control=100%).
left: semiconservative DNA synthesis
right: repair synthesis after an UV- dose of 100 erg/mm^2

Fig. 3 :

Arg - C

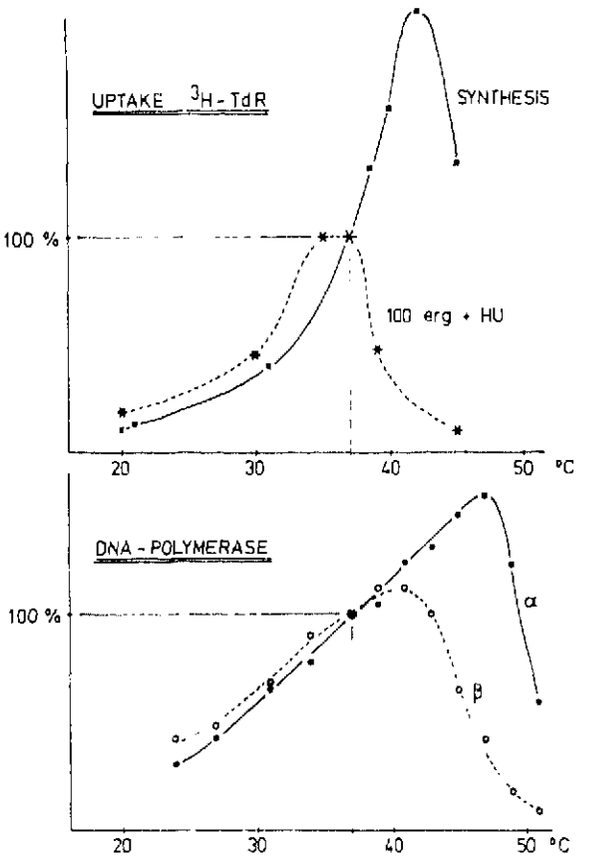


Inhibition by cytosine- arabinoside.

All values in relation to an untreated control (control=100%).

left: semiconservative DNA synthesis
right: repair synthesis after an UV-dose of 100 erg/mm^2

Fig. 4 :



All values in relation to the control at 37°C .

above: semiconservative DNA synthesis and repair synthesis at different temperatures
below: activity of DNA polymerase at different temperatures

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