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ENZYMATIC STUDIES OF RADIATION DAMAGE

MASTER

Progress Report

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Publications:

- C00-3225-30 Laskowski, M., Sr., Sulkowski, E., Duch, D.S., Richards, G.M., Kress, L.F., Dolapchiev, L.B., Sieliwanowicz, B., Yamamoto, M., Tutas, D.J., daRoza, D. and Pruch, J. "Nucleolytic Enzymes of the Venom of *Crotalus adamanteus*", in Animal, Plant, and Microbial Toxins, Vol. 1, ed. by A. Ohsaka, K. Hayashi and Yoshino Sawai, Plenum Press, New York, New York pp. 235-248 (1976).
- C00-3225-31 Kowalski, D., Kroeker, W.D. and Laskowski, M., Sr., "Mung Bean Nuclease I. Physical, Chemical and Catalytic Properties", *Biochemistry* 15, 4457-4463. (1976).
- C00-3225-32 Kroeker, W.D., Kowalski, D. and Laskowski, M., Sr., "Mung Bean Nuclease I. Terminally-Directed Hydrolysis of Native DNA", *Biochemistry* 15, 4463-4467 (1976).
- C00-3225-33 Kroeker, W.D. and Laskowski, M., Sr., "Polynucleotide Kinase: Functional Purification and Use in the Direct Kinetic Measurement of Single- and Double-Strand Cleavages of DNA by Restriction and Other Endonucleases of Limited Action", *Anal. Biochem.* 79, 63-72 (1977).
- C00-3225-34 Pritchard, A.E., Kowalski, David and Laskowski, M., Sr., "A Single-Strand Specific Endonuclease Activity of Venom Phosphodiesterase", *Fed. Proc.* 36, 907 (abst. 3352) (1977).

Summary

Papers on mung bean nuclease were discussed in detail in the last year report, even though only the abstracts were published at that time. Documents C00-3225-31 and C00-3225-32 are the full-length papers. Long delayed report on enzymes in venom was finally published (document C00-3225-30). The major accomplishments of the year were a new method (document C00-3225-33, and a totally unexpected discovery that venom phosphodiesterase belongs to the group of single-strand specific nucleases. It acts on viral duplex DNA by producing a series of shorter fragments of duplex DNA. It was also found that the relaxed circular DNA of PM2 virus, that was previously enzymatically ligated, may be opened by very high amounts of phosphodiesterase (document C00-3225-34). The new method (C00-3225-33) deals with a simplified way of preparing polynucleotide kinase and with fast removal of the excess of hot ATP used to label a nick in DNA. This is done by imbedding DNA within an agarose pellet, which retains labeled DNA, but allows the excess of ATP to diffuse out.

Research

Document C00-3225-30 is a long-delayed presentation of the results from our laboratory dealing with enzymes from venom. The presentation was made in September 1974 in Tokyo during the IV International Symposium on Toxinology. Since the time of the presentation, our laboratory has made considerable further progress.

As in the previous year, this year's major effort was directed at the elucidation of the mechanism of action of mung bean nuclease. Last year only the abstracts were published, this year two full-length papers (documents C00-3225-31 and C00-3225-32) were published in *Biochemistry*. The major accomplishments of this work was discussed in detail in the last year's report (C00-3225-24). Since that time, we have made several advances, but no additional published material appeared. The manuscript is in the process of preparation, and I believe it would be wiser to postpone the detailed discussion of these results until the final form of the manuscript is ready.

Further studies are carried out attempting to define more accurately the nature of the specific site. So far, all facts suggest that mung bean nuclease cleaves at the specific sites, which have single-strand character (permanent, like in damaged DNA, or temporary, like AT rich breathing regions and in the forking termini).

The study of the mechanism of the reaction would not have been possible without a simplified method to measure the number and nature of cleavages in the early stages of the reaction. Document C00-3225-33

describes the method to measure directly single- and double-strand cleavages by a rather simple procedure. Double-strand cleavages are distinguished from nicks by the well known action of alkaline phosphatase which removes monophosphates from the end of a fragment at 25°, but from the nick only at 65°. These two samples are then treated separately. To terminate the reaction alkaline phosphatase is destroyed by exposure to pH 12. Polynucleotide kinase and [γ ³²P] ATP are used to label the newly created 5'OH-termini. Up to now, the procedure is standard and has been used in many laboratories. There are, however, two serious drawbacks to this procedure. The preparation of highly purified polynucleotide kinase as described by Richardson *et al.* ((1968) *J. Biol. Chem.* 243, 4530-4542) is unusually touchy because of high losses of enzyme during the last two steps of the purification procedure. Commercially available samples are useless, because they are contaminated by one or more of interfering enzymes such as ATPase, endonuclease, 5'-exonuclease and 5'-terminal monophosphatase. To counteract this drawback a simple method for the preparation of partially purified polynucleotide kinase has been developed. The final product is about 1/4 as active as that of Richardson's preparation, but it is free from the interfering activities, and the yield is good. Omitting the details, the method utilizes steps worked out in the laboratories of Richardson, Khorana, Wu, with the final step worked out in our laboratory. To alleviate the second drawback, namely, a tiresome and incomplete dialysis of the excess of [γ ³²P] ATP, the diffusion from an agarose pellet was applied. After the kinase reaction is terminated, the reaction mixture is added to a drop of melted agarose which solidifies

on cooling to 25°. The solid agarose pellet is placed in buffer solution into which ATP, but not DNA rapidly diffuses. After three hours, the buffer is changed. After the additional two hours, pellet is recovered, rinsed in water and placed in a scintillation vial. The limit value of the remaining radioactivity varies from 0.003 to 0.005% of ATP used and depends on the preparation of [$\gamma^{32}\text{P}$] ATP. It is constant for each preparation, and presumably reflects degree of polymerization of the phosphate. This method allows same day accurate determination of a single cleavage per molecule of T₇ DNA.

The deviation from a close adherence to the plan occurred when it was found (document C00-3225-34) that venom phosphodiesterase (exonuclease) allowed to act on T₇ DNA does not produce the sticky ends. This enzyme has been studied in our laboratory for the past 20 years (mainly supported first by AEC, then by ERDA) and in 1974 (document C00-3225-16), a homogeneous preparation was obtained. In 1975, the method was further simplified (document C00-3225-28) and the presently available preparation fulfills criteria of physical homogeneity and is free from interfering activities. The enzyme is a classical exonuclease and acts on oligonucleotides by consecutive liberation of single monomers from the 3'-end, regardless of the position of the phosphoryl group (document NY0-3630-23). Many years ago, using less pure preparation (*J. Biol. Chem.* 236, 1130(1961)), it was found that viscosity changes only slightly when 10% of the native thymus DNA is converted to acid soluble products. It was, therefore, considered that the action on duplex DNA starts on both 3' ends toward the center and as a result long single-stranded tails should be formed. An experiment was performed using T₇ DNA that was

digested with venom phosphodiesterase to produce 15% acid soluble material, then the reaction was stopped by changing pH from 9 to 5 and mung bean nuclease was added. Instead of the expected doubling of acid soluble products, no further liberation was observed. This experiment showed that T₇ DNA is not attacked on both strands from the 3' ends by an exclusively exonucleolytic mechanism. The use of the technique of agarose gel electrophoresis and ethidium bromide staining confirmed this conclusion and showed that the action of venom phosphodiesterase on linear duplex DNA is endonucleolytic and produces a number of smaller linear duplex fragments. In this respect, it closely resembles the action of mung bean nuclease. It, therefore, strongly suggests that venom phosphodiesterase has a preference for single-stranded structures recognized by mung bean nuclease (AT rich, breathing regions). The more systematic study showed that even with the heat denatured T₇ DNA, some endonucleolytic cleavages occurred, but the relative rates of exonucleolytic cleavages (production of mononucleotides) increased only by a factor of 4.5 upon denaturation. The rate of cleavages required to relax the supercoil was two orders of magnitude slower. Nicked circular DNA was transferred to the linear form with a rate slower by another order of magnitude. However, a relaxed circular PM2 DNA that was enzymatically ligated to produce closed double-stranded circles was opened by venom phosphodiesterase with the rate that was 10⁶ slower. Of course, the probability of a contamination by a trace of different nuclease (endonuclease) has not been totally excluded. It does seem, however, very unlikely because the usual phosphodiesterase activity (measured on bis-p-nitrophenyl) and the ability to relax supercoiled

DNA go in parallel with respect to all types of inactivation. In addition, both activities appear at the same band on gel electrophoresis. The finding that a classical exonuclease has a similar single-strand specificity as mung bean nuclease was totally unexpected and suggests that not only the revision of nomenclature becomes timely, but also warrants re-examination of several properties of known nucleases on substrates more uniform than thymus DNA.