Splitting of the PypPu and PupPy Bonds by Deoxyribonucleases.* (31786)

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The specificity of the deoxyribonucleases has been under study for some years. The most extensive investigations have been directed at DNase I‡ which was early shown to have an endonucleolytic action resulting in the formation of polynucleotide fragments terminated in 5′-phosphates(1). Streptodornase has similarly been shown to be an endonuclease producing chains terminated in 5′-phosphates(2). Micrococcal nuclease(3) and DNase II from spleen(4) and thymus(5) are examples of another group of endonucleases which produce polynucleotide chains terminated in 3′-phosphates.

Laskowski and his coworkers have conducted a long series of investigations aimed at elucidating the specificity of the deoxyribonucleases. Direct analyses, by a variety of experimental techniques, of enzymic digests produced by these enzymes led to the suggestion that certain bonds were preferentially split. However, it was found that the mechanism of action was complex, and that specificity could change during the course of the reaction. These results have been the subject of reviews(6,7). Ralph *et al*(8) have shown that certain synthetic homologous polynucleotides were split by DNase I. Recently, an influence of the ionic environment on the enzymic splitting of DNA has been shown in several laboratories(9-12).

Progress in the understanding of the specificity of the deoxyribonucleases has been hampered by the lack of a method which measures the splitting of distinct chemical bonds in DNA.

In connection with the problem of characterization of isomeric dinucleotides(2), it was pointed out that the formic acid-diphenylamine reagent of Burton(13) could be used to distinguish between compounds of the structure pPypPu and pPupPy. The present communication is an extension of this work, and describes a method which permits direct measurement of the splitting of the PypPu and PupPy bonds by the appropriate deoxyribonuclease.

Materials and methods. The 4 possible nucleolytic cleavages are shown in Table I. The action of Burton's reagent on intact DNA releases Pi only from PupPu bonds, and in the case of calf thymus DNA amounts to about 27% of the total phosphorus content (13). Table I also shows that in the case of nucleases producing 5′-terminated chains, hydrolysis of the PypPu bond labilizes the internucleoside phosphate to the subsequent action of formic acid-diphenylamine so that Pi is now liberated. In the case of nucleases producing 3′-terminated chains, hydrolysis of the PupPy linkage similarly labilizes the internucleoside phosphate to the subsequent action of Burton's reagent. The amount of Pi liberated in excess of that produced from PupPu linkages by Burton's reagent alone is a measure of the splitting of the PypPu and PupPy bonds respectively.

The method simply consists of the enzymic digestion of DNA under the appropriate conditions of pH and metal activation. The reaction is terminated at the desired time by the addition of 2 volumes of 3% diphenylamine in concentrated formic acid. After 20 hours, the mixture is extracted with ether, and the aqueous layer lyophilized. The dry powder is dissolved in water, and analyses for Pi and total phosphorus carried out by the method of Fiske and Subbarow(14). Calf

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‡ DNase I: pancreatic deoxyribonuclease; Pu and Py: purine nucleoside and pyrimidine nucleoside respectively; A, G, C, T: nucleosides of adenine, guanine, cystosine, and thymine; letter p to left of nucleoside symbol indicates 5′-phosphate; letter p to right of nucleoside symbol indicates 3′-phosphate; d-: deoxysugar; Pi: orthophosphate. These are in accordance with customary abbreviations.

TABLE I. Liberation of Pi by Deoxyribonucleases and Burton's Reagent.

Bond	Pi liberated by Burton's reagent	Pi liberated by endonucleases producing 5′-terminated chains and Burton's reagent	Pi liberated by endonucleases producing 3′-terminated chains and Burton's reagent
....Pu ➡ p ⇨ Pu....	+	+	+
....Pu p Py....	—	—	+
....Py p Py....	—	—	—
....Py p Pu....	—	+	—

➡ Split by DNase I, streptodornase.
⇨ Split by DNase II, micrococcal nuclease.

thymus DNA was isolated by the method of Kay *et al*(15). Liberation of Pi from this sample by Burton's reagent was 27%. Similar values were obtained for commercial samples of high polymer calf thymus DNA purchased from Worthington Biochemical Corp. Control experiments showed that d-pC, d-pT, and d-Tp were stable to the action of formic acid diphenylamine, whereas Pi was quantitatively split from d-pA and d-pG. Only traces of Pi were detected in the enzymic digests prior to the addition of Burton's reagent.

Results and discussion. Table II shows that the attack on the PypPu bond by DNase I occurred to the extent of 1% of the phosphodiester bonds. It is known that calf thymus DNA is composed of 23% PypPu, 23% PupPy, 27% PypPy, and 27% PupPu linkages(16). Since total splits by DNase I approximate 25% of all bonds, random hydrolysis of the PypPu bond by this enzyme would result in the splitting of about 6% of phosphodiester linkages. Thus splitting of the PypPu bond by DNase I is neither random nor preferential, and accounts for only 4% of the entire enzymic event. Table II also shows that hydrolysis of the PupPy linkage by micrococcal nuclease occurred to the extent of 18% of the phosphodiester bonds. Since total cleavages by this enzyme approximate 50% of all bonds, random splitting of the PupPy bond should have occurred to the extent of 12%. These results indicate that the enzymic hydrolysis by this enzyme is not random, and that about 75% of all PupPy linkages are susceptible to the action of micrococcal nuclease. About 36% of the entire enzymic attack is accounted for by hydrolysis of PupPy bonds.

TABLE II. Splitting of the PypPu and PupPy Bonds by Deoxyribonucleases.

Deoxyribonuclease	Hydrolysis, % total phosphorus PypPu	PupPy
DNase I	1.0	
Micrococcal nuclease		18.0

Substrate was calf thymus DNA dissolved in water at a concentration of 2 mg per ml, and adjusted to the appropriate conditions of pH and ion content. All incubations were at 37° for 4 hr. Reaction volume was 10 ml. DNase I and micrococcal nuclease (crude) were purchased from the Worthington Biochemical Corp., Freehold, N. J. Digestion conditions were as follows: DNase I (20 μg): 0.1 M tris buffer, pH 7.4, containing 0.025 M $MgSO_4$; micrococcal nuclease (3.2 mg): 0.1 M tris buffer, pH 8.9, containing 0.01 M $CaCl_2$.

TABLE III. Splitting of the PupPy Bond by Micrococcal Nuclease.

Time, min	Hydrolysis, % total phosphorus
0	0
30	4.5
60	8.1

Digestion conditions: Calf thymus DNA at a concentration of 1 mg per ml in 0.1 M tris buffer, pH 8.9, containing 0.01 M $CaCl_2$. Reaction volume was 33 ml. Temperature 37°. Micrococcal nuclease (crude) 1.25 mg. Reaction terminated at time intervals by addition of 2 volumes of Burton's reagent.

Table III illustrates the hydrolysis of the PupPy bond by micrococcal nuclease as a function of time. It is apparent that there is a linear splitting of this bond during the

first hour of incubation. Further kinetic studies will be of interest to determine whether hydrolysis of this bond is a preferential event at some time during the enzymic degradation or represents a constant proportion of the total splits throughout the entire course of hydrolysis.

The method has yielded consistent and reproducible results on several samples of calf thymus DNA. There is, however, a large and irreducible blank value for Pi due to splitting of PupPu linkages by the Burton reagent itself. The minor degree of hydrolysis observed for the splitting of the PypPu linkage by DNase I was a consistent finding, and indicates that this bond is quite resistant to enzymic attack. It is of interest that splitting of the PypPu bond by DNase I in a sample of DNA isolated from *Clostridium perfringens* cells, where GC content is markedly different from that of calf thymus DNA, also occurred to the extent of only 1% of phosphodiester bonds.

Control studies have shown that the Fiske-Subbarow method is reliable for the estimation of Pi in the presence of pyrimidine polynucleotides. No splitting of phosphorus from the polynucleotides was noted, and the recovery of Pi added as internal standards was quantitative. Thus a single method can be used for measurement of both Pi and total phosphorus. The major methodologic pitfalls to be considered are the possible presence of contaminating phosphatases and exonucleases in the enzyme preparation. Both factors will tend to elevate values obtained by this method. Studies on other endonucleases are in progress, as well as the possible influence of the ionic environment on the hydrolysis of the PypPu and PupPy bonds.

Summary. A method has been developed which permits the direct measurement of the splitting of the PypPu bond in DNA by endonucleases producing polynucleotide chains terminated in 5′-phosphates, and of the PupPy bond by endonucleases producing polynucleotide chains terminated in 3′-phosphates.

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