A MAP OF THE PRODUCTS RESULTING FROM THE ACTION OF MICROCOCCAL NUCLEASE ON THYMUS DEOXYRIBONUCLEIC ACID AND ITS USE AS A GUIDE TO SPECIFICITY*

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A rapid and simple two-dimensional mapping procedure has recently been developed and applied to the resolution of digests of tobacco mosaic virus-ribonucleic acid (TMV-RNA) by pancreatic ribonuclease (RNase) (Rushizky and Knight, 1960a). This raised the question of the general applicability of the method and, in particular, the stability of mono-, di-, and tri-deoxyribonucleotides to the acidic solvents used in the electrophoretic and chromatographic steps. In answer to these points we report herein the partial resolution of the complex mixture of products resulting from the action of a highly purified nuclease on thymus deoxyribonucleic acid (DNA). The enzyme selected for this study was micrococcal nuclease (Cunningham _et al._, 1956) which has recently been investigated to establish the nature of the mono- and di-nucleotide products resulting from its action on DNA (Cunningham, 1958, 1959) and RNA (Cunningham, 1959; Reddi, 1959 a, b), respectively. The purification and specificity of this enzyme have also been under investigation in these laboratories (Dirksen, 1959; Reddi, 1959a) and it was readily available in a purified form which showed no activity towards di-(p-nitrophenyl) phosphate, nucleoside 2,3-cyclic phosphates, or mononucleotides. The substrate used was

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highly polymerized DNA from thymus isolated by the method of Schwander and Signer (1950) and preserved in citrate-sodium chloride solution at -15°.

About 60 mg. of DNA (as alcohol-precipitated fibers) was suspended in 46 ml. of 0.0025 M $CaCl_2$ adjusted to pH 9.0 and dissolved by stirring overnight at 5°. A typical "100% digest" was prepared as follows: Micrococcal nuclease (25 λ; sp. act. 40, Dirksen, 1959) was added to 15 ml. of the above stock DNA solution and the reaction was allowed to proceed at room temperature under nitrogen. Periodic additions of 0.4 N NaOH were made manually at the automatic titrator to maintain the pH at 8.5 ± 0.5. When 25% of the total bonds had been cleaved (8 hrs.), the reaction temperature was raised to 40° and another 10 λ of enzyme was added. After 4 hours the rate of alkali consumption had become very slow and a final addition of 10 λ of enzyme had no appreciable effect. The reaction was, therefore, brought to pH 9.0 and allowed to incubate at 37° under toluene for 10 hours. At the end of this time hydrolysis was assumed to be complete. From the amount of alkali consumed it could be calculated that 68% of the total bonds had been split. The solution was then lyophilyzed and the residue taken up in 2 ml. of water, giving a slightly cloudy solution. Aliquots of 0.3 ml. were withdrawn, applied to Whatman 3MM paper, and mapped as previously described (Rushizky and Knight, 1960a). The results are shown in Figure 1.

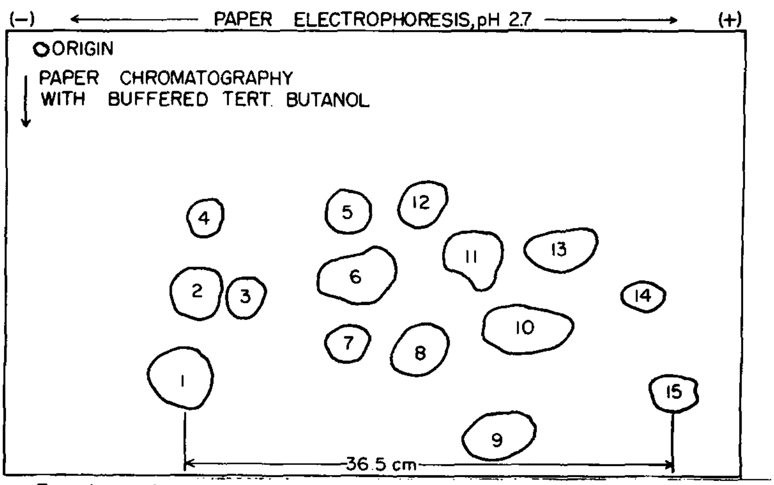

Fig. 1. Tracing of a print obtained from fractionated micrococcal nuclease digests of calf thymus DNA. (For the compounds present in the spots see Table I.)

In terms of optical density, about 95% of the total material applied was recovered in the fifteen spots shown in Fig. 1. This fact, plus our failure to observe either purines or depurinated fragments on the maps, attests to the stability of the deoxynucleotides in the solvents employed. The spots were eluted with water and the eluates subjected to paper chromatography with buffered ammonium sulfate (40 grams of ammonium sulfate dissolved in 100 ml. of 0.1 M sodium phosphate, pH 7.1). By this procedure it was shown that five of the spots contained two or more components. These, as well as the single components in the other spots, were characterized with respect to constituent bases using standard procedures (see Rushizky and Knight, 1960b). The relative amounts (T, i.e., thymidylic acid, is arbitrarily set equal to 100 moles) of the compounds so identified are shown in Table 1. In this table each 3'-nucleotide or 3'-nucleotide residue in an oligonucleotide is indicated by the first letter of the trivial name of the nucleotide. The fraction of the total bonds which would have to be broken to obtain this product distribution, namely 67%, is in good agreement with the value derived from the consumption of alkali during the enzymatic digestion of the DNA.

A "50% digest" was prepared by adding 25 λ of enzyme to 15 ml. of the stock DNA solution and maintaining the pH, as before, at pH 8.5 ± 0.5 with periodic additions of alkali. At the end of 10 hours at room temperature, when the amount of alkali consumed was one-half that required for the "100% digest", the reaction was terminated by acidifying to pH 4.5 with formic acid and freezing. After lyophilization, the digest was taken up in 2 ml. of water and aliquots mapped as before. While all of the products of the "100% digest" appeared in the mono-, di-, and tri-nucleotide region of the map, an appreciable amount of material (ca. 50% in O.D. units) in the "50% digest" had a negligible R_f in the chromatographic solvent and, therefore, remained as a band across the top of the filter paper "map". Those components found in the "100% digest" were usually found in the "50% digest" but the relative amounts differed. This is shown in Table 1 which lists the relative number

TABLE 1

Compounds identified in micrococcal nuclease digests of calf thymus DNA

Compound	Identifying number on map	Relative number of moles in the "100% digest"**	Moles of cpd in "50% digest" / Moles of cpd in "100% digest"
T	9	100.0	61
A	1	99.1	46
C	1	84.0	19
G	7	16.4	13
AT	8	5.7	46
TA	8	10.1	44
TT	15	13.5	40
AA	3	9.3	37
TG	10	34.3	29
AC	2	18.8	26
TC	8	25.7	25
(AG)*	6	27.6	23
CC	2	4.8	negligible
CG	6	4.3	negligible
GG	11	1.8	negligible
(TAG)	11	1.4	120
(TTG)	14	1.7	57
ACC	4	3.9	39
(ACG)	5	9.0	31
(TCG)	11	9.6	30
(TCC)	6	5.6	24
(AGG)	12	8.1	22
TGG	13	10.2	21

* The sequences of the constituent bases in the compounds in parentheses have not been determined.

** No correction has been made for hypochromicity. Therefore, base ratios calculated from these data will not be in exact accord with published values.

of moles of each compound found in the "100% digest", together with the percentage of each of these compounds found to be present in the "50% digest". We have arranged the mono-, di-, and tri-nucleotides so that the compounds which have been liberated to the largest extent in the "50% digest", relative to the amounts in the "100% digest", are listed first. (See Table 1, column 4). Those compounds appearing first in the mono-, di-, and tri-nucleotide groupings must be liberated more rapidly and, therefore, preferentially by the enzyme. When examined closely, the data in Table 1 are found to support the type of specificity for the enzyme discussed in the preceding paper (Dirksen and Dekker, 1960), namely an "A-T preference". It can be seen that A and T appear more rapidly than C and G and that dinucleotides comprised of A and/or T appear more rapidly than those that have one A (or T) and one G (or C) which in turn appear more rapidly than those made up of G and/or C. The same trend is perceptible in the tri-nucleotides, those with more G and/or C being more slowly released than those with less. Moreover, in those cases (see Table 1) in which we have determined the sequence of a major di- or tri-nucleotide component ($>$ 5% relative to T), the residue bearing the free 5'-hydroxyl group has been A or T. This is in agreement with the results of earlier studies (Cunningham, 1959). On the basis of these observations we have postulated a combined endo- and exo-esterase action of the enzyme, the first occurring in the region of high A and/or T concentration in a chain and the latter being less discriminatory. The complete fractionation of the digest and the identification and sequence determination of all major components is in progress to determine whether the common structural feature noted above is found in all the di- and tri-nucleotides liberated during the early phases of the digestion.

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