ENZYMIC DEGRADATION OF DESOXYRIBONUCLEIC ACID BY CRYSTALLINE DESOXYRIBONUCLEASE*

by

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In agreement with KUNITZ[2], CARTER AND COHN[3], and GORDON AND REICHARD[4], we have found that the degradation of desoxyribonucleic acid by crystalline desoxyribonuclease results in the formation of fragments, the great majority of which are larger than mononucleotides. By means of resin chromatography, it was possible to isolate a small fraction (amounting to not more than 2% of the total digest) exhibiting spectrophotometric characteristics which were different from those of the total digest and which indicated a predominance of cytidylic acid.

The present work was undertaken in the hope that at least a partial identification of the components of the fraction might be accomplished.

EXPERIMENTAL

Desoxyribonucleic acid was prepared according to the method of HAMMERSTEN[5]. Desoxyribonuclease, once recrystallized, was prepared according to the method of KUNITZ[6]. Digestion was carried out as follows: 0.5 gram of desoxyribonucleic acid was dissolved in 200 ml 0.2 *M* borate buffer pH 7.1, made 0.025 *M* with respect to magnesium sulfate; 2.0 mg of crystalline desoxyribonuclease were added, and the mixture was incubated for a period of five hours at 37°. At this time, all the phosphorus had become soluble in 7.5% trichloracetic acid[7]. A white precipitate, which formed during the enzymic digestion, was removed by centrifugation and discarded. The clear supernatant contained the split products of desoxyribonucleic acid.

The stability of this digest at room temperature and at different pH values was investigated. The criterion used was the appearance of inorganic phosphorus and/or reducing groups. The results are presented in Table I. No sign of hydrolysis could be detected upon exposure to hydrochloric acid at pH 2.4 over a period of 48 hours.

* This investigation was aided by a research grant (E 35) from the American Cancer Society, Inc. upon recommendation of the Committee on Growth of the National Research Council. A preliminary report has been published[1].

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§ Some of the data included in this report were taken from a thesis submitted by J. L. POTTER to the Graduate School of Marquette University in partial fulfillment of the degree of Master of Science.

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TABLE I

EFFECT OF ACID ON THE STABILITY OF THE DIGEST

pH	0-time		24 hrs		48 hrs		118 hrs		412 hrs	
	Inorg. P mg	Reduc. Value*	Inorg. P mg	Reduc. Palue	Inorg. P mg	Reduc. Value	Inorg. P mg	Reduc. Value	Inorg. P mg	Reduc. Value
4.4	0	0	0	0	0	5	0	7	—	—
3.2	0	0	0	0	0	4	0	2	—	—
2.4	0	0	0	0	0	7	0	8	1	102
1.8	0	0	0	23	0	48	0	94	—	—
1.2	0	0	0	90	0	92	0	112	3	108

* Expressed as mg of ribose

The resin used was Amberlite IR–400 which was prepared for chromatography by cycling a minimum of four times with hydrochloric acid and ammonium hydroxide. A column of this resin, 15 cm × 0.9 cm, was charged with 35 mg of the digest in 175 ml water adjusted to pH 6.5 with sodium hydroxide. Elution was carried out with hydrochloric acid pH 2.5 at a flow rate of 0.5 ml per minute. The effluent was collected until the spectrophotometric reading at 260 mμ no longer gave a significant value. The fraction thus obtained was designated as the "acid fraction" and accounted for no more than two per cent of the total digest expressed as phosphorus. Fig. 1 shows the spectrophotometric absorption curve of the acid fraction. The digest remaining on the resin could be partially eluted by either stronger acid or by alkali. Neither of these procedures

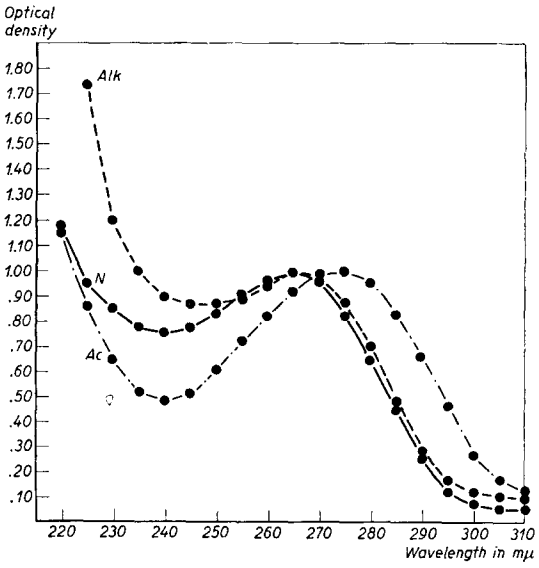

Fig. 1. Absorption curves of the acid fraction. The plotted points are relative values obtained by dividing the extinction value found at each wave length by the maximum extinction value in the particular medium[10]. Alk – alkaline; N – neutral; Ac – acid.

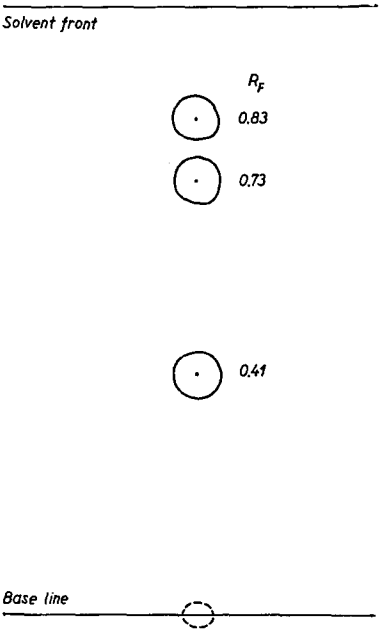

Fig. 2. Chromatogram of the acid fraction in alkaline phosphate-*iso*amyl alcohol.

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was deemed mild enough to exclude the possibility of further non-enzymic hydrolysis.

By the use of the paper chromatographic technique described by CARTER[8] for the resolution of ribonucleotides, the "acid fraction" was separated into three components, Fig. 2. The best separation was accomplished with alkaline phosphate-*iso*amyl alcohol, but it was also possible to effect a separation by means of acid phosphate.

The three spots were eluted and analyzed spectrophotometrically. The lowest spot (R_F 0.41) accounted for no more than 20% of the total material. As seen in Fig. 3, the curve resembles adenylic acid, but is sufficiently distorted to indicate contamination from either the paper or the resin. Figs. 4 and 5 illustrate the striking similarity between the curves obtained for the eluates from the middle spot (R_F 0.73) and upper spot (R_F 0.83). As Fig. 6 shows, the curves in acid media are practically superimposable. The E 280/260 ratio varied in different experiments from 1.7 to 2.3. In the majority of cases, this value was 2.1. Since the variation was observed in the eluates of both spots, it was attributed to an ultraviolet absorbing contaminant. The finding of two different spots with essentially identical spectrophotometric characteristics required further investigation. Two-dimensional chromatography in alkaline phosphate-*iso*amyl alcohol resulted in the maintenance of the approximate difference in the rate of movement of each spot. The R_F values in the first direction were 0.73 and 0.83, while in the second direction the R_F values were 0.70 and 0.79, thus confirming the existence of two separate entities.

The "acid fraction" contained neither inorganic phosphorus as determined by the method of FISKE-SUBBAROW[9], nor free bases as determined by the chromotographic technique of HOTCHKISS[10]. A complete hydrolysis with 6 *N* hydrochloric acid according to the method of DALY *et al.*[11] was carried out on an aliquot of the "acid fraction". The hydrolysate was chromatographed on paper according to the method of HOTCHKISS[10]. One spot was obtained with an R_F of 0.25 and there was a faint fluorescence in the position normally occupied by adenine. Table II shows the close conformity between the elution of the major spot of the hydrolysate and the values reported for cytosine by HOTCHKISS[10]. A highly concentrated sample of the "acid fraction" was chromatographed on paper using ammonium citrate-*iso*amyl alcohol at pH 9.6. In this solvent, adenylic acid appeared as a streak; and the two upper spots, consisting of the suspected isomers of cytidylic acids, appeared as one spot at an R_F of about 0.73. This spot was eluted, examined spectrophotometrically, and analyzed for total phosphorus. The ratios E 260/P and E 280/P (12) were calculated and were compared with similar ratios obtained for commercial ribocytidylic acid. (Table III). The results rule out a significant contamination with cytosine desoxyriboside. If such contamination had been present, the ratios E/P would have been higher then those determined for ribocytidylic acid.

TABLE II

CHARACTERISTICS OF R_F AND ABSORPTION VALUES

		Found	HOTCHKISS *Values for Cytosine*
R_F		0.25	0.26
N	275/265	0.82	0.82
N	230/245	1.4	1.46
OH	290/280	0.68	0.68

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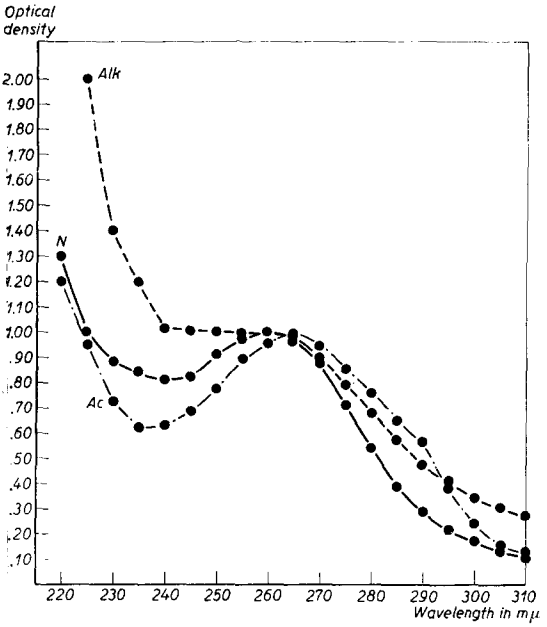

Fig. 3. Relative absorption curves of the eluate of the lowest spot; R_F 0.41. The spot was eluted overnight in distilled water. Alk – alkaline; N – neutral; Ac – acid.

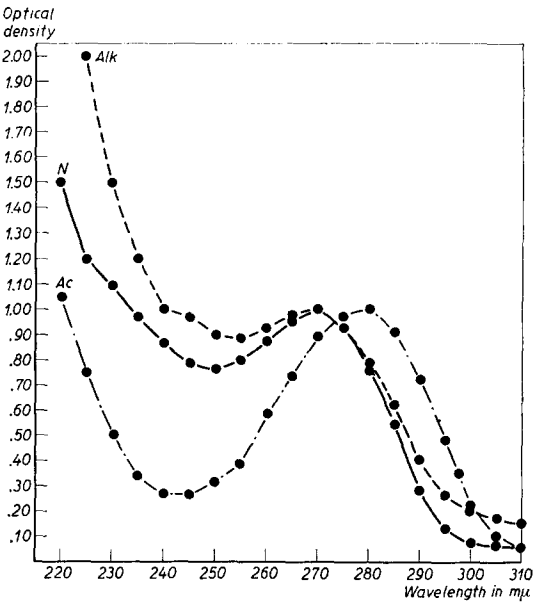

Fig. 4. Relative absorption curves of the middle spot; R_F 0.73. Same conditions.

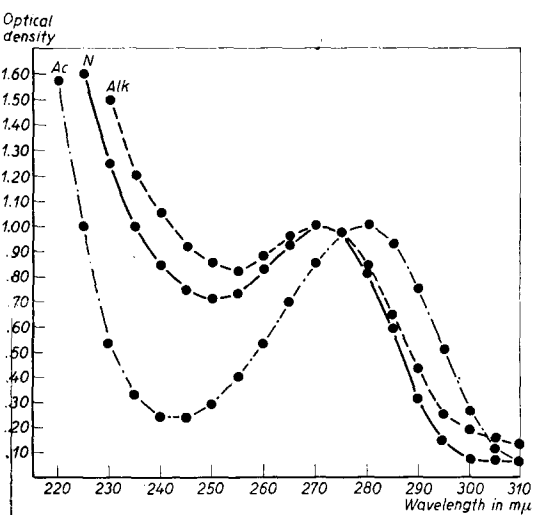

Fig. 5. Relative absorption curves of the upper spot; R_F 0.83.

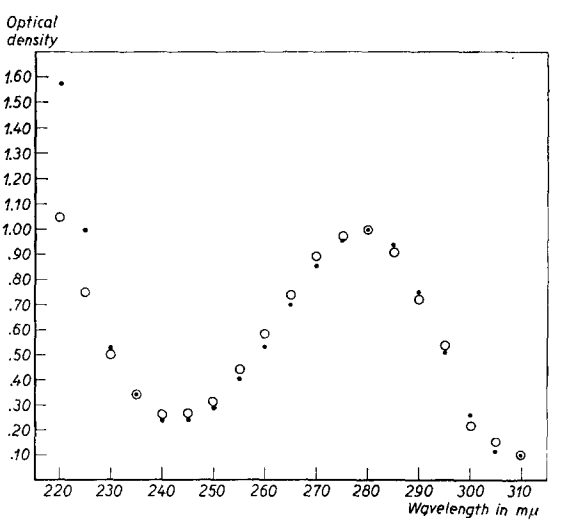

Fig. 6. Points obtained in acid medium for the eluate of the middle (R_F 0.73) — o —, and the upper (R_F 0.83) —●— spots.

TABLE III

Ribocytidylic acid (Commercial)	*Found in eluate*
$Ep = \frac{30.98 \times 18.6}{.098} = 5{,}900$ 260 mμ	4,400
$Ep = \frac{30.98 \times 36}{.098} = 11{,}400$ 280 mμ	9,300

An aliquot of the "acid fraction" was treated with acid phosphatase* according to the method of SCHMIDT *et al.*[13]: — 82% of the total phosphorus was liberated as inorganic. A similar aliquot was treated with 5-nucleotidase under the conditions described by HEPPEL AND HILMOE[14]**. This resulted in the liberation of 38% of the total phosphorus. The results of these experiments indicate that one of the observed components was 5-desoxycytidylic acid.

Essentially the same results were obtained with a sample of desoxyribonucleic acid kindly furnished by Dr A. E. MIRSKY. The "acid fraction" was present. The amount obtained was less than that from the HAMMARSTEN nucleic acid, but the spectral characteristics were identical. Three spots were obtained upon chromatography in alkaline phosphate-*iso*amyl alcohol. The upper two spots had identical spectra, corresponding to cytidylic acids.

DISCUSSION

It is of interest to note that the degradation of desoxyribonucleic acid by crystalline desoxyribonuclease results in the production of a mononucleotide in which the phosphate group appears on the C-5 of the sugar residue. This is in agreement with the recent work of CARTER[15] who demonstrated that the desoxynucleotides prepared by the use of two enzymes[16] were all 5-phosphodesoxyribosides. Futher attempts to characterize the second cytosine-containing component must await accumulation of much larger amounts of the material than have been available. At the present time, two alternatives remain: (a) the second spot is 3-desoxycytidylic acid, indicating the presence of isomers in the desoxyribonucleic acid; (b) this component is a dinucleotide composed of cytidylic acids.

The work of GORDON AND REICHARD[4] reached us after our experimental work had been completed. By use of electrophoresis in agar jelly, these authors were able to obtain several fractions composed of oligonucleotides but not sharply separated from each other. Our findings agreed with theirs in indicating that a fragment composed of cytidylic and adenylic acids was formed during the process of digestion by desoxyribonuclease. The difference between the findings in the two laboratories was that in ours a further degradation to at least one mononucleotide was observed.

In view of the above results it is interesting to speculate about the specificity of desoxyribonuclease. It appears that the linkage preferentially split must be associated with cytidylic acid. Since only a minor percentage of the total cytidylic acid present was liberated, it is obvious that additional pre-requisites for specificity exist.

The authors wish to thank Mr L. C. MASSOPUST for the drawings.

* We are greatly indebted to Dr G. SCHMIDT for supplying us with acid phosphatase.
** We are greatly indebted to Dr L. A. HEPPEL for supplying us with 5-nucleotidase.

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ADDENDUM

While this manuscript was in press, a letter to the editors (R. L. SINSHEIMER AND J. F. KOERNER, *J. Am. Chem. Soc.*, 74 (1952) 283) appeared. These authors identified di-desoxycytidylic acid in the products of digestion of desoxyribonucleic acid by desoxyribonuclease. In view of their finding, additional support is given to the alternative we proposed suggesting that the second component is di-desoxycytidylic acid.

SUMMARY

Small amounts of 5-desoxycytidylic acid were identified in the digest of desoxyribonucleic acid with desoxyribonuclease. Since only mild conditions have been used during the chromatographic isolation, it is believed that this mononucleotide resulted from the direct action of the enzyme and not from subsequent treatment of the digest. In addition, a second substance having almost identical spectrophotometric properties has been observed. It has not been definitely identified at present, but it is believed to be either 3-desoxycytidylic acid or a polymer of desoxycytidylic acids.

RÉSUMÉ

Nous avons identifié de faibles quantités d'acide 5-désoxycytidylique dans de l'acide désoxyribonucléique digéré par la désoxyribonucléase. Des conditions douces ayant été employées lors de l'isolement chromatographique, nous sommes d'avis que ce mononucléotide résulte de l'action directe de l'enzyme et non du traitement subséquent. De plus, nous avons observé une seconde substance ayant des propriétés spectrophotométriques presque identiques. Cette substance n'a pas encore été identifiée d'une facon définitive, mais nous pensons qu'il s'agit d'acide 3-désoxycytidylique ou d'un polymère d'acides désoxycytidyliques.

ZUSAMMENFASSUNG

In mit Desoxyribonuclease verdauter Desoxyribonucleinsäure wurden geringe Mengen von 5-Desoxycytidylsäure identifiziert. Da bei der chromatographischen Isolierung nur milde Bedingungen angewendet wurden, wird angenommen, dass dieses Mononucleotid seine Entstehung der direkten Enzymwirkung und nicht der auf diese folgenden Behandlung verdankt. Ausserdem wurde eine zweite Substanz beobachtet, welche beinahe identische spektrophotometrische Eigenschaften besitzt. Diese wurde bisher nicht endgültig identifiziert, doch wird angenommen, dass es sich um 3-Desoxycytidylsäure und um polymere Desoxycytidylsäuren handelt.

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Received October 11th, 1951