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In retrospect: a commentary by

Erwin Chargaff

New York, NY (U.S.A.)

on 'Studies on the structure of ribonucleic acids'
by B. Magasanik and E. Chargaff
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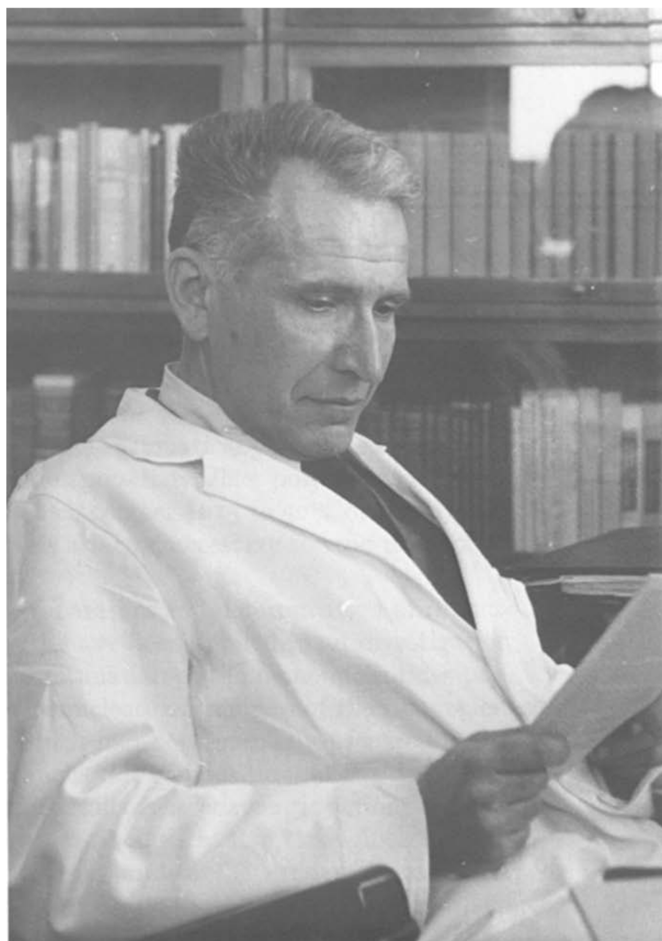
When I glance over those unassuming pages written a long time ago I cannot help both admiring and deploring the great advances that our science has made in the last thirty or forty years. Admiring: because, if it is the task of scientific research to produce new knowledge about what the scientist calls nature, we have learned a great deal in that period. Deploring: because, while in its beginnings research was a modest home industry in which a single craftsman labored in intimate contact with one or two apprentices, it has now become a 'high-tech' undertaking, in which factories for the technological manufacture of results produce items of knowledge – many trivial, some important – of which one can say, paradoxically, that the more are being made, the more expensive they become. The cost of research has outrun its value for mankind. The tension introduced into the life of the individual researcher has distorted his satisfaction with the discovery. More often than not he is a member of a very large team, fighting his way between nonrecognition and overassertion. The gratification he derives resembles that of an ancient Egyptian dragging a huge stone block towards a pharaoh's pyramid.

The state of affairs that, I fear, I am one of the few to deplore can be resumed in the form of a question: how many biochemical papers published forty years ago still are alive, as any other intellectual production is likely to be? The answer will be "Very few", many fewer than would have been the case before Big Science took over. One should have thought that a scientific paper, at any rate in the exact sciences, published nearly forty years ago, would remain citable even now and that its results, for what they are worth, would be as 'true' now as they were when first revealed. This may still be the case in a few disciplines, but surely no longer in biochemistry. A finding related to inorganic or organic chemistry may have a life expectancy of a hundred years or more; and if the sciences really were what they



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claim to be it should actually last forever. But in biochemistry, I am afraid, the life expectancy is down to three or five years. This is, however, not the place in which to discuss the philosophical and sociological causes that have led to so remarkable a decline in the

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vitality and survival power of much of present research.

The word 'Biochemistry' is a hybrid formation, similar to the designations of many other scientific disciplines. But whereas, say, in paleozoology the prefix merely qualifies, mildly, the basic noun, 'bio-' is an unusually weighty prefix which must overwhelm whatever it is affixed to; and the tension thus produced has attended the science of biochemistry from its very beginning. During most of its duration the practitioners of this science possessed, however, a thorough training in chemistry: they were essentially chemists devoting their knowledge to the problems of living tissues. But in the last twenty-five years this has changed and biochemistry has been made into the handmaiden of the new and ill-defined science of molecular biology of which it is not easy to say in what, if anything, its practitioners are trained thoroughly. Some people may reject all that as nomenclatural quibbles, denying that biochemistry has been displaced by molecular biology of which they maintain that it is the same old hybrid seen from behind. Those would still accept the jocular definition that I proposed many years ago: Molecular biology is the practice of biochemistry without a licence.

Be that as it may, it must be regarded as a great pity that the beautiful and satisfactory science of biochemistry has been superseded, prematurely, by that piquant ragout whose wholesomeness is not attested to by the increasing number of swindles to which it is giving rise. This shift has had the consequence that a great number of valid biochemical problems are no longer pursued, since, considered as unfashionable, they are not funded. It is regrettable that philosophers, historians and sociologists of science rarely look at the budget. Twenty morons at the right places can kill a science.

Correspondence: E. Chargaff, 350 Central Park West, Apt. 13-G, New York, NY 10025, U.S.A.

STUDIES ON THE STRUCTURE OF RIBONUCLEIC ACIDS*

by

BORIS MAGASANIK** AND ERWIN CHARGAFF

Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York 32, N.Y. (U.S.A.)

The composition of several ribonucleic acids has, in the recent past, been investigated frequently[1–4]. A precise knowledge of the proportions in which the several components occur in a nucleic acid is, of course, one of the requisites for an understanding of its structure. (See the discussion in[5]). But detailed information, as yet almost unavailable, about the order and the manner in which the mononucleotides are linked to form a molecule of nucleic acid will be required in addition. One of the procedures applicable to the elucidation of this problem is the controlled, stepwise degradation of the polymers by chemical or enzymatic means and the determination of the complete composition of the split products formed at the various stages. This approach has recently been followed in this laboratory with respect to the action of desoxyribonuclease on desoxypentose nucleic acids[6,7]. For the investigation of ribonucleic acids in this manner, two depolymerizing agents are available: alkali, which under proper conditions produces quantitative cleavage to mononucleotides[2,4]; and the enzyme ribonuclease, which breaks part of the nucleic acid to small fragments, considered to consist predominantly of pyrimidine nucleotides[8] or of a mixture of all four mononucleotides[9]. A recent report indicated that the composition of the enzymatically produced dialyzable fragments changed in the course of digestion[10]. A non-dialyzable, polymerized residue is known to remain after enzymatic digestion which comprises 15 to 50% of the original nucleic acid and is rich in purines[8,11]; it contains a factor necessary for the production of streptolysin S by streptococci[12,13].

In the present study, the methods for the separation and determination of ribonucleotides, recently developed in this laboratory[14], have been applied to an investigation of the following problems: a. identification and estimation of the mononucleotides produced by the exhaustive action of crystalline ribonuclease on samples of the ribonucleic acids from both yeast and pig liver, some of which had been specially prepared under mild conditions[§]; b. determination of the total nucleotide composition[4] of the dialyzable and non-dialyzable fractions formed from the nucleic acids by enzymatic action; c. study of the mechanism of action of dilute alkali on yeast ribonucleic acid through an investigation of the composition of the fragments released, and of the residues retained, at different time intervals, A number of spectroscopic observations, related to the problem of nucleic acid structure, are also discussed.

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** Present address, Department of Bacteriology and Immunology, Harvard Medical School, Boston, Massachusetts.

§ We are very grateful to Mr D. ELSON AND Mrs. C. GREEN for help with these preparations.

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EXPERIMENTAL

MATERIALS

Yeast Ribose Nucleic Acid

Three preparations of this substance, designated Y-1, Y-2, and Y-3, were used. Preparations Y-1 and Y-2 were described in a previous publication[4] where they were listed as No. 2 and 3 respectively.

The isolation of Preparation Y-3 from yeast, ground in a wet crushing mill[15], followed in some respects the procedure employed for Preparation No. 4 described previously[4]. The nucleic acid was, however, precipitated with neutral rather than acidic ethanol and, thus, isolated as the sodium salt, without having been in contact with acid or alkali in the course of its preparation. The material was found to contain 8.5% of desoxypentose nucleic acid, as determined by the diphenylamine reaction[16]; it was, therefore, purified by dialysis at room temperature in the presence of crystalline desoxyribonuclease[17] and Mg ion, and recovered by precipitation with ethanol. The air-dried preparation Y-3 (yield about 0.15%) contained 6.4% P and 1.7% of desoxypentose nucleic acid.

Pig Liver Ribose Nucleic Acid

Two preparations were studied. Preparation L-1 was described as No. 6 in a previous publication[4]. Preparation L-2 was isolated in a similar way, but with the modifications (including the treatment with desoxyribonuclease) described above for Y-3. It was free of desoxypentose nucleic acid and contained 5.1% P (air-dried). Comparison of the analytical results will show that the ribonucleic acid specimens prepared as the sodium salts were more contaminated with non-phosphorylated material than were those isolated as the free acids; but it may be presumed that they were less degraded.

The total nucleotide composition of the various ribonucleic acid preparations studied here is presented later in connection with the degradation experiments.

Enzyme Preparations

The crystalline preparations of pancreatic ribonuclease and desoxyribonuclease were supplied by the Worthington Biochemical Laboratory, Freehold, New Jersey.

METHODS

Analytical Procedures

Phosphorus was determined by a colorimetric procedure[18]. *Mononucleotides* were separated and estimated, as described previously[14]. The same procedures served for the determination of the *nucleotide composition* of the various fractions following their hydrolysis with dilute alkali ("Procedure 1", p. 57 of a previous publication[4]). The nucleotide separations were carried out in the "acid system"[14]. The measurements of absorption in the ultraviolet were carried out in M phosphate buffer of p_H 7.1 in a Beckman ultraviolet quartz spectrophotometer.

Dialysis

All dialysis experiments were carried out in small bags made of cellulose casing ("No Jax", Visking Corporation, Chicago).

Degradation by Ribonuclease

Between 1 and 3 ml of 5 to 9% solutions of nucleic acid in 0.2 *M* acetate buffer of p_H 6.0 together with 1 mg of ribonuclease were introduced into small dialysis bags which were placed in test tubes containing 4 to 8 ml of the same buffer. Another 1 mg portion of ribonuclease was dissolved in the outside fluid, in order to ensure the complete enzymatic digestion of the dialyzable components.

The dialysis was carried out at room temperature, with shaking, and was allowed to proceed until the estimation of the phosphorus contents and the ultraviolet extinctions of the dialysates indicated that diffusion had practically come to a standstill. Following removal of the dialysis bag, the outside fluid served for nucleotide determinations, if necessary after concentration *in vacuo*. For purposes of calculation it was assumed that the dialyzable components were, at this point, equally distributed between the inside and outside dialysis fluids.

For the isolation of the dialysis residue, the dialysis was continued for 24 hours at room temperature against two changes of 1 liter each of the acetate buffer. The outside fluids were discarded; the contents of the dialysis bag were concentrated by evaporation to a volume of 0.5 to 1 ml, and transferred quantitatively to a 5 or 10 ml volumetric flask before the phosphorus contents, ultraviolet extinction, and nucleotide composition were determined.

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Degradation by Alkali

About 5% solutions of the nucleic acid, dissolved in dilute ammonia, were brought to p_H 13.5 by the careful addition of 33% NaOH, adjusted to a measured volume and kept at 30°. At definite time intervals, from 15 minutes to 48 hours after the start of the experiment, aliquot portions of the solution were withdrawn and neutralized, and their contents in free mononucleotides determined. Other portions of these aliquots were subjected to dialysis at room temperature against two 1 liter portions of distilled water for 24 hours. The dialysis residues were recovered by evaporation in the frozen state, redissolved in water and precipitated by the addition of 3 volumes of ethanol that had been made 0.05 *N* with respect to HCl. The usual analyses were then carried out.

Spectroscopy of Nucleic Acid Fractions

For the determination of the ultraviolet absorption of the various nucleic acid fractions and of their alkaline hydrolysates, aliquot portions of the solutions of known phosphorus content, ranging in volume from 0.01 to 0.05 ml and containing from 100 to 500 γ of nucleic acid P, were added, by means of an accurate micrometric burette, to 4.0 ml of *M* phosphate buffer of p_H 7.1.

RESULTS

Preliminary Dialysis

Preparation Y-2 (from yeast) and L-1 (from pig liver) had not been dialyzed in the course of their isolation and purification. Prior to the study of the enzymatic degradation of these substances, it was therefore necessary to ascertain the amount and the composition of their dialyzable components. After about 40 hours dialysis in the case of Y-2, and 18 hours in the case of L-1, against a small volume of 0.2 *M* acetate buffer (p_H 6.0) at room temperature no further increase in dialyzable material could be detected. The composition of the diffusible fractions, which amounted to about 20% in both nucleic acid preparations, is shown in Table I. Chromatography[14] of the dialysates, prior to alkaline hydrolysis to mononucleotides[4], revealed only slow-moving components that formed long streaks on the paper, beginning at the starting point. It is likely that the dialysates consisted of polynucleotides of varying size. The ultraviolet absorption characteristics of these fractions and of their alkaline hydrolysates are summarized in Table VIII.

Action of Ribonuclease

The preparations Y-3 from yeast and L-2 from liver had been dialyzed in the course

TABLE I

DIALYZABLE AND NONDIALYZABLE FRACTIONS OF PENTOSE NUCLEIC ACID PREPARATIONS Y-2 AND L-1

Preparation	Fraction	Phosphorus		Nucleotide composition (as moles per 100 moles nucleotide)				Phosphorus balance (P accounted for as nucleotides) per cent organic P in fraction
		mg	per cent of starting material	Guanylic acid	Adenylic acid	Cytidylic acid	Uridylic acid	
Y-2	Y-2 Starting	8.4	100	28	27	23	22	93
	Y-2D Dialyzable	1.5	18	28	28	18	26	83
	Y-2R Nondialyzable	5.8	69	28	26	22	24	87
L-1	L-1 Starting	14.5	100	33	20	32	15	83
	L-1D Dialyzable	3.5	24	28	29	20	23	88
	L-1R Nondialyzable	9.4	65	38	16	33	13	82

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of their isolation. They were used for the enzymatic degradation experiments immediately after their preparation without further preliminary dialysis. The yeast preparation Y-2 did contain a diffusible fraction which, however, as shown in Table I, did not differ in composition from the nondialyzable fraction; for this reason, this nucleic acid was also employed without preliminary dialysis. The liver preparation L-1, on the other hand, included a differently composed dialyzable fraction (Table I). Therefore, the nondialyzable fraction of this preparation, designated L-1R in Table I, was employed as the substrate in the enzymatic experiments. The course of the dialyses is illustrated in Fig. 1.

When the dialysates, collected after about 20 hours of combined enzymatic digestion and dialysis, were subjected to paper chromatography[14], ultraviolet absorbing spots, corresponding in position and ultraviolet spectrum to the mononucleotides cytidylic acid and uridylic acid, could be detected. Neither adenylic acid nor guanylic acid was found.

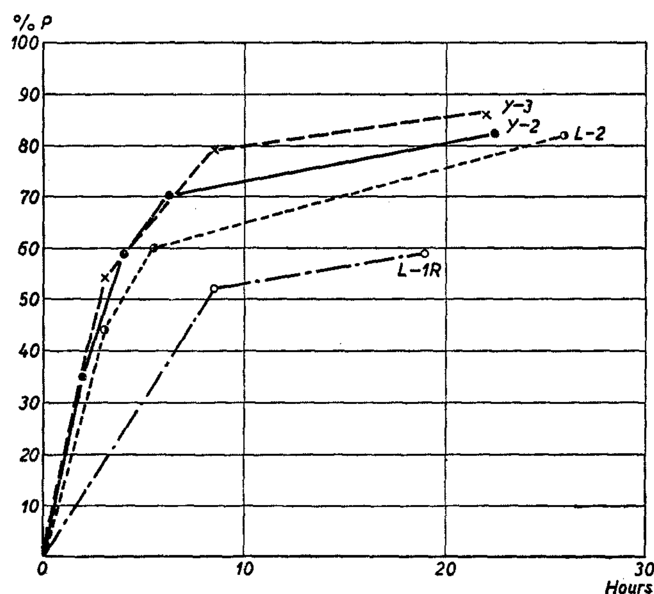

Fig. 1. Rate of dialysis of breakdown products of ribonucleic acids from yeast (Preparations Y-2 and Y-3) and from pig liver (Preparations L-1R and L-2) in presence of ribonuclease. The dialyzable phosphorus as per cent of total nucleic acid P present is plotted against time

In addition, the chromatograms showed three fairly well separated zones of slower-moving, ultraviolet absorbing components, located between the starting point and the position of uridylic acid. In the case of the yeast preparations, no absorbing material remaining at the starting point could be detected. With the pig liver preparations, on the other hand, a small quantity of unidentified material that did not migrate in the solvent system used was found.

Table II provides a summary of the findings; it presents the proportions of mononucleotides and total dialyzable nucleotides liberated by the action of ribonuclease.

The composition of the dialysis residues, collected after additional dialysis, as described above, is summarized in Table III. In the light of the discussion presented at a previous occasion ([4] p. 63) it may be doubted that the values for the small amounts of uridylic acid found in the dialysis residues have more than qualitative significance.

In order to facilitate the comparison of the nucleotide composition of the nucleic

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TABLE II

ACTION OF RIBONUCLEASE ON PENTOSE NUCLEIC ACIDS: COMPOSITION OF DIALYZABLE DEGRADATION PRODUCTS*

Preparation	Fraction	Phosphorus		Nucleotide composition (as moles per 100 moles nucleotide in starting material)					Phosphorus balance (P accounted for as nucleotides) per cent organic P in fraction
		mg	per cent of starting material	Guanylic acid	Adenylic acid	Cytidylic acid	Uridylic acid	Total	
Y-2	Starting	16.7	100	28	27	23	22	100	93
	Dialyzable (total nucleotides)	13.8	83	17	19	18	22	76	85
	Dialyzable (mononucleotides)			0	0	14	21	35	
Y-3	Starting	7.0	100	30	21	23	26	100	88
	Dialyzable (total nucleotides)	6.0	86	18	15	19	26	78	80
	Dialyzable (mononucleotides)			0	0	14	19	33	
L-1R	Starting	7.6	100	38	16	33	13	100	81
	Dialyzable (total nucleotides)	4.5	59	17	9	22	12	60	83
	Dialyzable (mononucleotides)			0	0	18	7	25	
L-2	Starting	2.8	100	36	19	29	16	100	97
	Dialyzable (total nucleotides)	2.3	82	18	14	26	15	73	87
	Dialyzable (mononucleotides)			0	0	18	10	28	

* The periods at which the collection of the dialyzable fractions was terminated were 22.5, 23, 26, and 22 hours for Preparations Y-2, Y-3, L-1R, and L-2 respectively.

acids serving as the substrates and of the dialysis residues resulting from the action of ribonuclease, the molar relationships, listed in Table IV, have been expressed as proportions of individual nucleotide per 10 moles of adenylic acid. The distribution of combined and of free mononucleotides in the fractions released in a dialyzable form by the action of ribonuclease is presented in Table V.

The ultraviolet spectra, before and after alkaline hydrolysis, of Preparations Y-3 and L-2 and of the several fractions derived from them are illustrated in Fig. 2. The expression $\varepsilon(P)$ has been defined previously[19]. Extinction data for all fractions are pre-

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TABLE III

ACTION OF RIBONUCLEASE ON PENTOSE NUCLEIC ACIDS: COMPOSITION OF DIALYSIS RESIDUES*

Preparation serving as starting material	Phosphorus		Nucleotide composition (as moles per 100 moles nucleotide)				Phosphorus balance (P accounted for as nucleotides) per cent organic P in fraction
	mg	per cent of starting material	Guanylic acid	Adenylic acid	Cytidylic acid	Uridylic acid	
Y-2	0.9	5	63	27	7	3	94
Y-3	0.5	7	66	18	8	8	84
L-1R	1.3**	17	65	15	14	6	64
L-2	0.24	9	71	15	0	14	76

* Following the collection of the dialyzable degradation products, summarized in Table II, the nondialyzable fractions were subjected to an additional dialysis, as described in the text, before being recovered.

** Includes about 0.5 mg of desoxypentose nucleic acid P.

TABLE IV

ACTION OF RIBONUCLEASE ON PENTOSE NUCLEIC ACIDS: MOLAR RELATIONSHIPS IN NUCLEOTIDE COMPOSITION OF STARTING MATERIALS AND DIALYSIS RESIDUES

Preparation	Fraction	Guanylic acid	Adenylic acid	Cytidylic acid	Uridylic acid	Purines / Pyrimidines
Y-2	Starting	10	10	9	8	1.2
	Dialysis residue	23	10	3	1	8.3
Y-3	Starting	14	10	11	12	1.0
	Dialysis residue	37	10	4	4	5.9
L-1R	Starting	24	10	21	8	1.2
	Dialysis residue	43	10	9	4	4.1
L-2	Starting	19	10	15	8	1.3
	Dialysis residue	47	10	0	9	6.3

TABLE V

ACTION OF RIBONUCLEASE ON PENTOSE NUCLEIC ACIDS: DISTRIBUTION OF COMBINED AND FREE MONONUCLEOTIDES IN DIALYZABLE DEGRADATION PRODUCTS

Preparation	Nucleotides in dialyzable fraction (as per cent of individual nucleotide in starting material)							
	Guanylic acid		Adenylic acid		Cytidylic acid		Uridylic acid	
	Total	Free	Total	Free	Total	Free	Total	Free
Y-2	61	0	70	0	78	61	100	95
Y-3	60	0	71	0	83	61	100	73
L-1R	45	0	56	0	67	56	92	54
L-2	50	0	74	0	90	62	94	63

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sented in Table VIII, with the exception of the dialysis residue resulting from the enzymatic digestion of L-1R which was too heavily contaminated with desoxypentose nucleic acid.

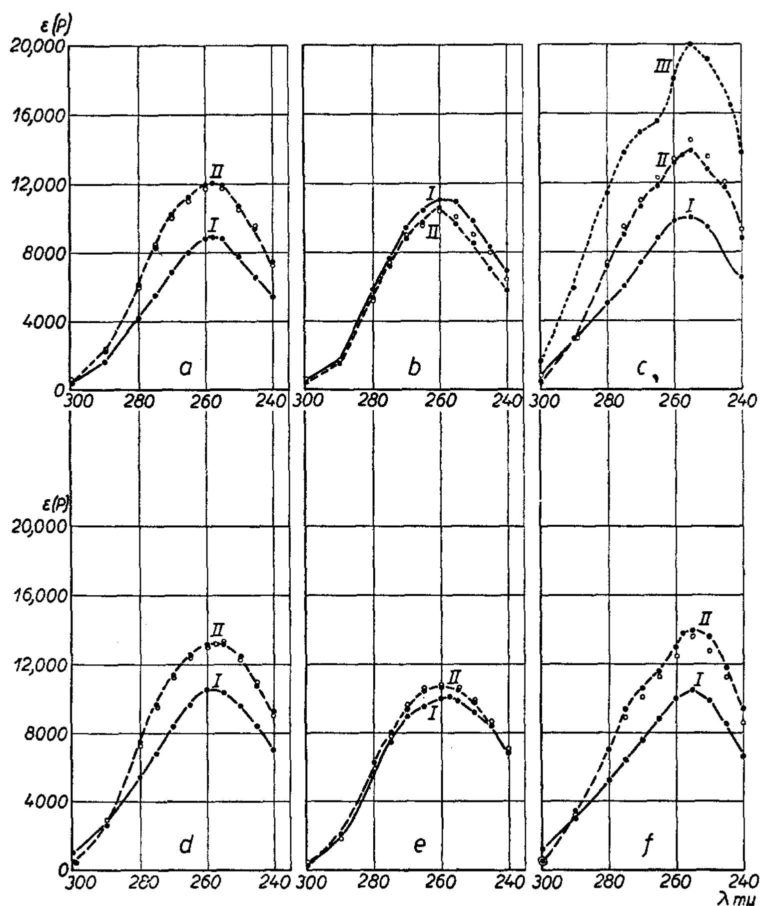

Fig. 2. Ultraviolet absorption spectra of ribonucleic acids from yeast (Preparation Y-3) and from pig liver (Preparation L-2) and of their enzymatic breakdown products before and after alkaline hydrolysis. Curve I (solid line) represents, in all cases, the intact fraction, Curve II the alkaline hydrolysate (full circle, found values; empty circles, values calculated from nucleotide composition). Curve III in Fig. 2c illustrates the spectrum of guanylic acid. Preparation Y-3: a. Intact nucleic acid; b. Dialyzable fraction; c. Nondialyzable fraction. Preparation L-2: d. Intact nucleic acid; e. Dialyzable fraction; f. Nondialyzable fraction

Alkaline Hydrolysis

These experiments were carried out with the yeast preparation Y-1. The results are summarized in Table VI as regards the course of liberation of mononucleotides; in Table VII the composition of the polynucleotide residues remaining after brief action of alkali is presented; optical data are included in Table VIII.

The chromatograms of the fractions produced by the brief action of alkali (15 to 225 minutes), which are summarized in Table VI, showed, in addition to the spots corresponding to the mononucleotides, several other adsorption zones that were probably due to oligonucleotides. One such zone was located a little above the region containing

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guanylic and uridylic acids. A slight contamination by that material of the extracts of these nucleotides may be responsible for the somewhat high recoveries of uridylic acid recorded in these experiments. Another zone, located between cytidylic and adenylic acids, was close enough to the former to render impossible its quantitative determination.

The hydrolysis proceeded very rapidly; the polynucleotide residues remaining after 15 and 30 minutes (Table VII) amounted to only 2 and 1% respectively of the starting material.

TABLE VI

ACTION OF ALKALI ON YEAST RIBONUCLEIC ACID: COURSE OF LIBERATION OF MONONUCLEOTIDES FROM PREPARATION Y-1

Experiment No.	Duration of alkaline hydrolysis minutes	Mononucleotides, found (as moles per 100 moles nucleotide in starting material)				
		Guanylic acid	Adenylic acid	Cytidylic acid	Uridylic acid	Total
1	30	18	8	*	28	54
	90	26	18	*	26	70
	225	27	27	*	24	78
2	15	19	9	*	26	54
	35	23	15	*	23	61
	2880	29	30	21	20	100

* In these experiments, cytidylic acid could not be determined.

TABLE VII

ACTION OF ALKALI ON YEAST RIBONUCLEIC ACID: COMPOSITION OF POLYNUCLEOTIDE RESIDUES REMAINING AFTER INCOMPLETE ALKALINE HYDROLYSIS OF PREPARATION Y-1

Experiment No.	Duration of alkaline hydrolysis minutes	Nucleotide composition (as moles per 100 moles nucleotide)			
		Guanylic acid	Adenylic acid	Cytidylic acid	Uridylic acid
2	0	29	30	21	20
	15	24	38	26	12
	35	21	45	24	10

DISCUSSION

The reliability of the analytical methods and the adequacy of the preparations employed should be considered first. The accuracy of the chromatographic procedures for the quantitative study of the nucleotide composition of pentose nucleic acids has been discussed in previous publications[4,14], where it was shown that the recovery of nucleotides, computed on the basis of ultraviolet extinction, after the alkaline hydrolysis of the nucleic acids was close to 100%. Similar recoveries were recorded in all nucleotide

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TABLE VIII

ULTRAVIOLET EXTINCTION OF PENTOSE NUCLEIC ACIDS AND DERIVED FRACTIONS BEFORE AND AFTER ALKALINE HYDROLYSIS

Experiment No.	Preparation	Fraction	Described in Table	Extinction at 260 mμ		
				Starting ε (P)	Alkaline hydrolysate ε (P)	Increase per cent
1	Y-1	Intact	VII	9,700	12,100	25
2		Residue (15 minutes alkaline hydrolysis)	VII	9,900	10,700	8
3		Residue (35 minutes alkaline hydrolysis)	VII	9,400	10,500	12
4	Y-2	Intact	I, II	10,000	12,400	24
5		Dialyzable, nonenzymatic (Y-2D)	I	11,800	11,700	-1
6		Nondialyzable, nonenzymatic (Y-2R)	I	8,600	11,500	33
7		Dialyzable, enzymatic	II	10,800	11,300	5
8		Nondialyzable, enzymatic	III	11,000	13,700	25
9	Y-3	Intact	II	8,700	11,900	37
10		Dialyzable, enzymatic	II	11,000	10,300	-6
11		Nondialyzable, enzymatic	III	9,800	13,100	34
12	L-1	Intact	I	8,600	11,100	29
13		Dialyzable, nonenzymatic (L-1D)	I	10,600	11,800	11
14		Nondialyzable, nonenzymatic (L-1R)	I	8,300	10,500	27
15		Dialyzable, enzymatic	II	10,400	11,100	7
16	L-2	Intact	II	10,400	13,200	27
17		Dialyzable, enzymatic	II	10,000	10,700	7
18		Nondialyzable, enzymatic	III	10,000	13,000	30

analyses reported here, as can, for instance, be seen in Fig. 2, where the ultraviolet extinction coefficients, expressed as ε (P)[19], of the nucleic acid hydrolysates produced by alkali are compared with the spectra constructed on the basis of the nucleotide composition determined by chromatography. The total recoveries thus seem to be reliable, but some of the figures relating to nucleotides present in very small amounts in certain fractions, particularly some of the values for uridylic acid, may be subject to greater errors, as may be the figures derived from experiments in which mixtures of mono- and oligonucleotides were subjected to direct chromatography. The comparison of the ultraviolet spectra of the separated components with those of the corresponding authentic mononucleotides excluded, however, major errors arising from this source.

The quality of the ribonucleic acid preparations available at present and the problems relating to their homogeneity have been considered at previous occasions[1,4,5]. Preparations that, in the course of their isolation, have not been subjected to dialysis appear to contain at least two principal fractions differing materially in molecular size (Table I). In the case of one of the yeast preparations employed in the present study, Preparation Y-2, no marked differences in the composition of the dialyzable and non-

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dialyzable polynucleotide fractions were observed. The ribonucleic acid from pig liver, Preparation L-1, on the other hand, consisted of a slowly diffusible polynucleotide fraction similar in composition to yeast ribonucleic acid and of a nondialyzable component in which the deviations from equimolar proportions of the constituents, found in the original preparation, were accentuated. The other preparations had been dialyzed immediately before use. In view of the lack of proper criteria for testing, no statement can be made at present as to the degree of intactness and homogeneity of the ribonucleic acids.

The attack of ribonuclease on a ribonucleic acid produces three types of compounds: mononucleotides, dialyzable polynucleotides, and nondiffusible cores. A decisive interpretation of findings gathered from the reaction of an agent of unknown specificity with a substrate of complex and partially undetermined structure is, of course, impossible. Nevertheless, certain typical features can be distinguished from what may be accidental. The over-all effects of ribonuclease presented here, especially in Tables II to V and in Fig. 1 and 2, were, in fact, remarkably uniform, when the diversities in source, preparation, and total composition of the substrates are considered. It may be concluded that all ribonucleic acids employed had elements of a structural organization in common that allowed the enzyme to degrade them in an analogous manner.

In view of the high concentration of both substrate and enzyme employed in the present study it may be assumed that the enzymatic reaction proceeded to completion very rapidly and that the rate at which nucleic acid P appeared in the dialysate depended on the quantity and the molecular size of the breakdown products rather than on the velocity of their formation. As is shown in Fig. 1, a relatively short period of very rapid dialysis, resulting in the appearance of 60 to 70% of the nucleic acid P in the dialysate, was followed abruptly by a period of extremely slow diffusion. Chromatography of the dialysates, collected during the first 20 hours and consisting preponderantly of material accumulated in the rapid phase of dialysis, showed them to be composed of nucleotides capable of migrating on filter paper irrigated with the isobutyric acid system. The action of the enzyme resulted in the cleavage of 60 to 80% of the nucleic acid in the form of small oligonucleotides and of mononucleotides; the rest of the substrate remained in a polymerized form.

The inspection of Tables II and V will show that only pyrimidine nucleotides appeared as mononucleotides in the dialyzable fractions split off under the influence of the enzyme. These mononucleotides accounted for one third and one quarter, respectively, of the nucleotides present in the yeast and pig liver preparations. The remainder of the rapidly dialyzing fractions, comprising combined nucleotides, consisted in the main of purine nucleotides together with a small proportion of pyrimidine compounds. The guanylic acid portion of the dialysates accounted in all cases for 17 to 18 mole per cent of the starting materials (Table II); it amounted, consequently, to a smaller proportion of the total guanylic acid content of the liver preparations that were initially particularly rich in this nucleotide (Table V). Almost the entire uridylic acid of the nucleic acids studied was found in the dialysates.

The composition of the dialysis residues at the time of the collection of the dialysates serving for the analyses summarized in Table II has not been given here, in order to conserve space; it can easily be computed from the data presented in that Table. In order to recover the polynucleotide fragments of the nucleic acids that could be regarded as truly resistant to ribonuclease (Tables III and IV), a supplementary dialysis was

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applied during which a material change in the composition of the dialysis residues took place. The final dialysis residues consisted to about two thirds of guanylic acid (Table III). The fractions of intermediate size, lost during the second stage of dialysis, contained 40 to 50% of guanylic acid in the case of the yeast preparations, somewhat more in the others; they were free of uridylic acid. The observation that the "limit ribonucleic acids" produced by, and resistant to, ribonuclease are composed of fragments of different size and composition may find its explanation in the initial lack of homogeneity of the starting preparations.

The bearing of the findings here presented on the problems of ribonuclease specificity and ribonucleic acid structure can now be considered briefly. The following generalizations emerge: a. Three groups of enzymatic breakdown products can be distinguished, *viz,* mononucleotides, diffusible oligo- or polynucleotides, and nondiffusible residues; b. The mononucleotide fraction is exclusively composed of pyrimidine nucleotides; c. The dialyzable polynucleotides consist of a large proportion of the total adenylic acid present in the nucleic acid specimen, of a somewhat smaller proportion of the total guanylic acid, and of small quantities of cytidylic and uridylic acids; d. In the non-dialyzable cores, the ratio of guanylic acid to the sum of the other nucleotides is not far from 2; e. The alkaline hydrolysis of the cores (as well as of the intact nucleic acids), but not of the dialyzable degradation products, produces a strong hyperchromic effect in the ultraviolet.

As regards the specificity of the pancreatic ribonuclease, it is clear that this enzyme is able to break bonds involving pyrimidine nucleotides, since pyrimidine mononucleotides in large amounts make their appearance in the digestion mixtures. It is, however, not capable of cleaving all such bonds, since some pyrimidine nucleotides in a combined form are found in both the dialysates and the "limit nucleic acids". For a more precise definition of the enzymatic specificity two hypotheses could be considered: a. The enzyme can only hydrolyze bonds between pyrimidine nucleotides; b. The enzyme can hydrolyze all bonds except those linking guanylic acid to other nucleotides.

The first supposition would lead to the conclusion that in the ribonucleic acids a large proportion of the pyrimidine nucleotides are linked to each other, uninterrupted by purine nucleotides. The remainder of the pyrimidine nucleotides, still adhering to the enzymatically produced polynucleotide fragments, would be assumed to occur as end groups next to purine nucleotides or, flanked by them, within the molecules.

The second hypothesis concerning the specificity of the enzyme, namely that it is unable to split internucleotide links in which guanylic acid takes part, is perhaps somewhat more plausible. It would satisfactorily explain certain striking regularities observed with respect to the distribution of guanylic acid. All fractions that are resistant to the enzyme are particularly rich in this nucleotide (Table III); and it may be inferred that the very high guanylic acid content of the ribonucleic acid from pancreas is not unrelated to the reported resistance of this nucleic acid to ribonuclease[3]. The structures of the enzyme-resistant cores of the ribonucleic acids thus could be regarded as represented by formulae I or II or, more probably, by a combination of both. (G denotes guanylic acid, X any of the other nucleotides). The analytical results for the "limit nucleic acids" reported in Tables III and IV favor a structure in which the formulation I preponderates. Polymers of this type, tending in composition towards a polyguanylic acid, could be assumed to form the core or backbone of the original pentose nucleic acid molecules. It is perhaps significant that in all ribonucleic acids of known composi-

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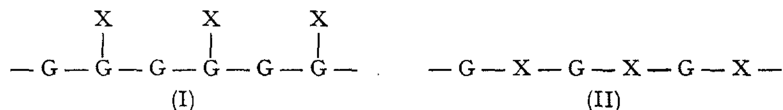
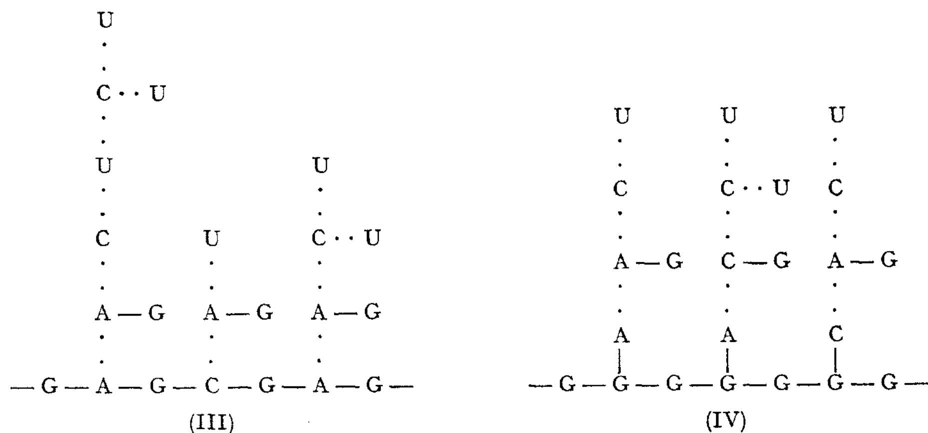
tion the concentration of guanylic acid, the major constituent of the core, equals, and in many cases surpasses, that of any other nucleotide[4]. It is worthy of note that in the desoxyribonucleic acid of calf thymus, on the other hand, the composition of the core has been found to tend towards that of a polyadenylic acid[7].



It now remains to consider the manner in which the nucleotides, rendered diffusible under the influence of ribonuclease, had originally been attached to the core. That branching is one of the features of the structure of ribonucleic acids has been made likely by the results of GULLAND and his associates[20,21] and of KHOUVINE AND GRÉGOIRE[22]. Structures III and IV are examples of polynucleotides in which branches rich in pyrimidine nucleotides are attached to a core comprising about 30 mole per cent of the entire nucleotides. (The abbreviations G, A, C, U stand for guanylic, adenylic, cytidylic, uridylic acids respectively. The bonds that are hydrolyzed by ribonuclease are indicated by broken lines, on the assumption, discussed above, that the enzyme is able to split all internucleotide bonds except those through which guanylic acid is linked to another nucleotide). In these formulations adenylic acid was assumed always to be bound to guanylic acid, in view of the absence of adenylic acid from the enzymatically produced mononucleotides.



It will be noted that the view set forth here concerning the action of ribonuclease, which assumes it to consist in the degradation of side chains, leaving behind an impregnable polynucleotide core or a mixture of such cores, whose composition will depend upon the initial structure of the particular nucleic acid, is not dissimilar from current conceptions of the mode of action of amylases and phosphorylases. A conclusive test of the validity of this view, particularly with regard to the structural restrictions on the extent of the enzymatic hydrolysis of nucleic acids, will depend on the availability of synthetic substrates, especially mixed di- and trinucleotides of suitable composition.

The results of the alkaline hydrolysis experiments afford a test of the plausibility of the proposed nucleic acid structures. It may be assumed that the agent causing hydrolysis in this case, namely the hydroxyl ion, has equal access to all bonds susceptible of cleavage, so that the rate at which mononucleotides are liberated will essentially

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depend on whether one, two or three bonds have to be broken, in order to effect their release. The experiments summarized in Table VI revealed, in fact, marked differences in the rates of liberation of the mononucleotides. Uridylic acid is set free most readily, as short an exposure to alkali as 15 minutes being sufficient for its complete liberation. Adenylic acid is released at a comparatively very slow rate, guanylic acid about twice as fast as adenylic acid. Cytidylic acid could not be measured in these experiments.

The minute fractions of the original nucleic acids, remaining in a non-dialyzable form after brief, incomplete alkaline hydrolysis (Table VII), are richer in adenylic acid and poorer in uridylic acid than the starting preparations. If the liberation rate of individual mononucleotides by alkali depends on the number of bonds that must be split simultaneously, as postulated above, a nucleic acid of the structure exemplified by Formulation III would give rise to residues of such composition.

An interesting phenomenon can be observed when the ultraviolet absorption spectra of the intact pentose nucleic acid fractions and of their enzymatic degradation products are compared with the spectra of the mononucleotide mixtures obtained from them by the action of alkali. As is shown in Table VIII (Experiments 1, 4, 9, 12, 16) and in Fig. 2a and 2d, the ultraviolet extinctions of the original nucleic acid specimens are considerably lower than those of a mixture of mononucleotides of the same composition, when the measurements are carried out under identical conditions in M phosphate buffer of p_H 7.1*.

It is obvious that the association of pentose mononucleotides to form highly polymerized nucleic acids introduces a new quality into the optical behavior of these substances; it is accompanied by a suppression of certain chromophores, *i.e.* by a decrease in the number or the types of resonating structures, capable of absorbing ultraviolet light of a wavelength around 260 mμ. While the level of polymerization above which this effect becomes noticeable cannot yet be strictly defined, it is clear that the effect is not due to the simple union of a few mononucleotides, since nucleic acid fractions consisting of polynucleotides, but small enough to be dialyzable, showed almost the same extinction as the mononucleotide mixtures to which they could be hydrolyzed by alkali (Table VIII, Experiments 5 and 13). Similarly, in the experiments with ribonuclease the dialyzable fractions of the reaction products showed no increase in extinction when subjected to alkaline hydrolysis (Table VIII, Experiments 7, 10, 15, 17; Fig. 2b and 2e), whereas hydrolysis of the nondialyzable cores was accompanied by strong hyperchromic effects (Table VIII, Experiments 8, 11, 18), the resulting absorption curves differing in both height and shape from those of the unhydrolyzed substances (Fig. 2c and 2f).

That the pyrimidine nucleotides are in all likelihood not involved in this phenomenon may be gathered from the fact that the enzyme-resistant cores, which contain only a small proportion of pyrimidine nucleotides (Table III), show it to the same extent as the intact nucleic acids. In Fig. 2c the absorption spectra of the core of Preparation Y-3 before and after alkaline hydrolysis are compared with the spectrum of guanylic acid; Fig. 2f illustrates the spectra of the core of Preparation L-2. Guanylic acid has, in addition to the major absorption band with a maximum at 254 mμ, a weaker, partly

* For the time being, only increases larger than 20% of the original extinction will be considered as truly significant. It is not certain whether small extinction increases reported in the recent literature, *e.g.* in the spectra of tobacco mosaic virus nucleic acid read at p_H 11.9[23] and of yeast ribonucleic acid following treatment with perchloric acid[24], are related to the group of phenomena discussed here.

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obscured band at 275 mμ. Indications of a similar band appear in the alkaline hydrolysates of the cores, although it is partly covered by the absorption of the other nucleotides, while the spectral curves of the unhydrolyzed cores are smooth and almost symmetrical with no suggestion of an absorption band in that region. It may be tentatively concluded from these observations that it is the resonance of the guanine ring which is hindered in the highly polymerized molecules. Additional support for this assumption may be seen in the magnitude of the extinction increase on hydrolysis exhibited by the enzyme-resistant cores which consist to two thirds or more of guanylic acid. Particle size in itself is, however, not the sole determinant of this partial suppression of resonance. The nondialyzable residues from incomplete alkaline hydrolysis, in which adenylic acid was the major constituent (Table VII), failed to exhibit the hyperchromic effect (Table VIII, Experiments 2 and 3).

The increases in absorption intensity observed after the alkaline hydrolysis of the intact nucleic acids and of the nondialyzable residues from the enzymatic digestion are of the same magnitude (Table VIII, Experiments 1, 4, 8, 9, 11, 12, 16, 18). Since the absorption of the enzymatic dialysates undergoes only negligible changes under the same conditions (Table VIII, Experiments 7, 10, 15, 17), some of the bonds responsible for the lowered extinction of the original polymer must have been broken by the enzyme. This inference is in keeping with the results obtained by CHANTRENNE *et al.*[25] by microdilatometry, which were interpreted as showing ribonuclease to act in two ways: as a depolymerase and as a phosphatase. There is, however, no need to assume a direct action of the enzyme on any but ribose-phosphate bonds. As was recently pointed out in connection with a discussion of the action mechanism of desoxyribonuclease[7], an automatic snapping of secondary valence bonds could, in a macromolecule, very well be the consequence of the enzymatic rupture of covalent links.

The spectroscopic observations presented here may be regarded as an indication of the presence in highly polymerized pentose nucleic acids of linkages involving the purine or pyrimidine rings in addition to the ribose to phosphoric acid bonds. Guanylic acid, when present as a major component in certain polymerized structures, appears primarily concerned in this phenomenon which finds its expression in a partial suppression of resonance. In Table IX several events, pertinent to the present discussion, that result from the degradation of desoxypentose and of pentose nucleic acids, respectively, are listed and compared. The degradation of desoxypentose nucleic acids has been discussed in greater detail in recent publications from this laboratory[7,30]. It will be noticed that the degradation by enzymes or alkali of the two types of nucleic acid appears, in some respects, to proceed by remarkably different routes*. To what extent some of these differences will be reconciled, when better preparations of pentose nucleic acids become available, remains to be seen. It is quite possible that secondary valence bonds of the type postulated as necessary for the maintenance of the macromolecular structure of desoxypentose nucleic acids[29,30] will be found also to occur in intact pentose nucleic acids** and that it is to the existence of such supplementary linkages and to

* One perplexing fact is the failure of the treatment with alkali at pH 13 of several desoxypentose nucleic acids (from calf and pig thymus, pig liver, and salmon sperm) to cause a hyperchromic effect in the ultraviolet[27], whereas desoxyribonuclease has been reported to produce it[17]. One should have imagined that if hydrogen bonds are broken as the direct or indirect result of enzymatic action, this would surely also have been the case with alkali.

** In line with what has been pointed out in a previous publication ([4], Footnote 6) it may be assumed that the ε (P) of intact pentose nucleic acid preparations will be found considerably lower than the values of 9,000 to 10,000 usually encountered.

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their interference with the resonating system of the purine and pyrimidine rings that the hyperchromic effects discussed here are attributable.

We should like to record with gratitude the assistance of Miss RUTH DONIGER in the course of these experiments.

TABLE IX

CONSEQUENCES OF NUCLEIC ACID DEGRADATION

Degrading agent	Desoxypentose nucleic acids	Pentose nucleic acids*
Desoxyribonuclease	Intensification of absorption in ultraviolet[17]	—
Desoxyribonuclease	Nondialyzable core, relatively much richer in adenylic acid[6,7]	—
Desoxyribonuclease	No mononucleotides formed[6,7]	—
Ribonuclease	—	No intensification of absorption in ultraviolet[26]
Ribonuclease	—	Nondialyzable core, relatively much richer in guanylic acid
Ribonuclease	—	Pyrimidine mononucleotides formed
Alkali (p_H 13)	No intensification of absorption in ultraviolet[27]	Strong intensification of absorption in ultraviolet
Alkali (p_H 13)	Depolymerization to nondialyzable polynucleotides[7] without change in composition[28]	Brief action leaves small quantity of nondialyzable residue, relatively richer in adenylic acid
Alkali (p_H 13)	No dialyzable breakdown products formed[7]	Prolonged action effects quantitative hydrolysis to mononucleotides[4]
Alkali (p_H 12)	Rupture of hydrogen bonds, unblocking of enolic hydroxyls and amino groups, disappearance of anomalous titration curve[29]	**

* Most pentose nucleic acid preparations available certainly are inferior in quality to those of the desoxypentose nucleic acids.

** Yeast ribonucleic acid has been found to give normal titration curves and no indications of blocking of hydroxyls and amino groups were obtained[20]. Because of the lability to alkali of the pentose nucleic acids these findings are, however, not strictly comparable to those on desoxypentose nucleic acids[29].

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SUMMARY

The course of action of crystalline ribonuclease on the ribonucleic acids from yeast and from pig liver was studied with the use of chromatographic and spectroscopic procedures. The findings formed the basis for the development of a number of conceptions as to the structure of the macromolecular pentose nucleic acids.

About 60 to 70 mole per cent of the nucleotides present in the initial substrates were liberated by enzymatic action as rapidly dialyzable nucleotides of low molecular weight. This fraction consisted of free cytidylic and uridylic acids (comprising a high proportion of the total pyrimidine nucleotides) and of combined purine and pyrimidine nucleotides.

All nucleic acid preparations yielded a nondialyzable residue, resistant to enzymatic attack, which was found to consist to about two thirds of guanylic acid and of varying amounts of the other nucleotides.

The exposure of pentose nucleic acids to weak alkali results ultimately, as is known, in their complete hydrolysis to mononucleotides. In the case of yeast ribonucleic acid, it could be shown that this rapid hydrolysis does not proceed at random, but that the several mononucleotides are released at widely differing rates, uridylic acid being the first and adenylic acid the last to be detached.

The spectroscopic observations centered around a comparison of the ultraviolet extinctions of the intact nucleic acids with those of the enzymatic breakdown products and of the mononucleotide mixtures to which they gave rise on alkaline hydrolysis. At 260 mμ, the extinctions of the intact compounds and of their nondialyzable cores, produced by ribonuclease, were very considerably lower (25 to 37%) than those of the corresponding mononucleotide mixtures. The ultraviolet absorption spectra of the fractions rendered dialyzable by the enzyme showed, however, no such extinction decrease.

The implications of these findings for the problems of ribonuclease specificity and ribonucleic acid structure are discussed and possible patterns of nucleotide sequence in backbone and sidechains of a branched macromolecule are considered.

RÉSUMÉ

Nous avons étudié la marche de l'action de la ribonucléase crystallisée sur les acides ribonucléiques de levure et de foie de porc à l'aide de méthodes chromatographiques et spectroscopiques. En nous basant sur les résultats obtenus nous avons développé une série d'idées sur la structure des acides nucléiques pentosiques macromoléculaires.

Environ 60 à 70% (en mols) des nucléotides contenus dans le substrat initial étaient libéré par action enzymatique sous forme de nucléotides à poids moléculaire bas et rapidement dialysables. Cette fraction consistait en acides cytidylique et uridylique libres (représentant une proportion élevée de la quantité totale de nucléotides pyrimidiques) et en nucléotides puriques et pyrimidiques combinés.

Toutes les préparations d'acides nucléiques donnèrent naissance à un résidu non-dialysable, résistant aux enzymes, qui se composait d'environ deux tiers d'acide guanylique et de quantités variables d'autres nucléotides.

On sait que le traitement des acides nucléiques pentosiques par l'alcali faible résulte finalement en leur hydrolyse complète et la formation de mononucléotides. Pour l'acide ribonucléique de levure il a été montré que cette hydrolyse rapide ne procède pas au hasard, mais que divers mononucléotides sont libérés à des vitesses très différentes, l'acide uridylique étant libéré le premier et l'acide adénylique le dernier.

Le sujet essentiel de l'étude spectroscopique consistait en une comparaison des valeurs d'extinction dans l'u.v. des acides nucléiques intactes avec celles des produits de dégradation enzymatique et des mélanges de mononucléotides qui en résultent lors de l'hydrolyse alcaline. A 260 mμ les valeurs d'extinction des composés intactes et des résidus non-dialysables résultant de la digestion par la ribonucléase étaient considérablement plus basses (25 à 37%) que celles des mélanges de nucléotides correspondants. Cependant, les spectres d'absorption u.v. des fractions rendues dialysables par l'enzyme ne montraient pas une telle diminution de l'intensité de l'absorption.

Nous avons discuté les conclusions à tirer de ces résultats au sujet de la spécificité de la ribonucléase et de la structure des acides ribonucléiques. Diverses dispositions et ordres possibles des nucléotides dans la chaîne principale et les chaînes latérales d'une macromolécule ramifiée ont été pris en considération.

ZUSAMMENFASSUNG

Der Verlauf der Einwirkung der kristallisierten Ribonuklease auf die Ribonukleinsäuren aus Hefe und Schweineleber wurde mit Hilfe chromatographischer und spektroskopischer Methoden

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untersucht. Auf Grundlage der Ergebnisse wurde eine Reihe von Vorstellungen über die Struktur der makromolekularen Pentosenukleinsäuren entwickelt.

Etwa 60 bis 70 Molprozent der im Ausgangssubstrat vorhandenen Nukleotide wurden durch das Enzym als rasch dialysierende, niedrigmolekulare Nukleotide freigesetzt. Diese Fraktion bestand aus freier Cytidyl- und Uridylsäure, die den Hauptteil der gesamten Pyrimidinnukleotide vorstellten, und aus gebundenen Purin- und Pyrimidinnukleotiden.

Alle Nukleinsäurepräparate lieferten einen nicht-dialysierbaren, enzymfesten Rückstand, welcher zu etwa zwei Drittel aus Guanylsäure bestand und aus wechselnden Mengen der anderen Nukleotide.

Bekanntlich führt die Behandlung der Pentosenukleinsäuren mit schwachen Alkalien schliesslich zur vollständigen Hydrolyse zu Mononukleotiden. Für die Ribonukleinsäure aus Hefe konnte gezeigt werden, dass der Verlauf dieser schnellen Hydrolyse nicht dem Zufalle ausgesetzt ist,sondern dass die verschiedenen Mononukleotide mit weitgehend verschiedener Geschwindigkeit freigesetzt werden: Uridylsäure zuerst und Adenylsäure zuletzt.

Den Hauptgegenstand der spektroskopischen Untersuchungen bildete ein Vergleich der Absorptionsintensitäten im Ultraviolett der ursprünglichen Nukleinsäuren mit denen der enzymatischen Abbauprodukte und der Mononukleotidgemische, die aus ihnen bei alkalischer Hydrolyse entstehen. Bei 260 mμ waren die Absorptionsintensitäten der intakten Verbindungen und der nicht-dialysierbaren Verdauungsrückstände viel niedriger (25–37%) als die der entsprechenden Mononukleotidgemische. Hingegen wiesen die Ultraviolettspektren der durch das Enzym in dialysierbarer Form abgespaltenen Fraktionen keine solche Intensitätsverminderung auf.

Die Folgerungen, die sich aus diesen Ergebnissen mit Bezug auf die Probleme der Spezifizität der Ribonuklease und der Struktur der Ribonukleinsäuren ergeben, wurden erörtert und verschiedene Möglichkeiten für die Anordnung und Reihenfolge der Nukleotide im Rückgrat und in den Seitenketten eines verzweigten Makromoleküls in Betracht gezogen.

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