THE COMPOSITION OF THE PENTOSE NUCLEIC ACIDS OF YEAST AND PANCREAS*

By ERNST VISCHER† and ERWIN CHARGAFF

(From the Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York)

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The historically understandable attempts to simplify the problem of nucleic acid structure by the formulation of the tetranucleotide hypothesis have found their classical exposition in Levene's monograph of 1931 (1). The same tendencies are expressed, less precisely, in statements found in many text-books according to which the nucleic acid unit, having a molecular weight of about 1200, contains four different bases (two purines and two pyrimidines) in equimolecular proportions. With the growing recognition of the complex and macromolecular character of the nucleic acids the necessity for the postulation of these hypothetical units vanished and it became possible to consider nucleic acids as substances comparable to the proteins in intricacy and, perhaps, even in specificity.

Our present knowledge of the structure of nucleic acids has been reviewed repeatedly in the recent past (2–4). Other relevant aspects also have been considered (5, 6).

The method for the quantitative estimation of individual purines and pyrimidines in mixtures of these bases, presented in the preceding paper (7), has made possible a new approach to the study of the composition of nucleic acids. The present communication describes the application of these procedures to a study of the distribution of the nitrogenous constituents of the pentose nucleic acids of yeast and pig pancreas and includes a consideration of aspects related to this problem, such as the mechanisms of the acid hydrolysis of these compounds. Other accounts, to be submitted shortly, will deal with the composition of desoxypentose nucleic acids. The results reported here will, later in this paper, be correlated with the findings of previous workers.

EXPERIMENTAL

Material

Ribonucleic Acid of Yeast—The purification procedure used was a modification of that of Fletcher *et al.* (8). 15 gm. of yeast nucleic acid (Merck)

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† Swiss-American Student Exchange Fellow.

were dissolved in 400 cc. of water by the addition of 15 cc. of 2 N ammonia. The filtered solution (pH 6.2) was dropped, with mechanical stirring, into 860 cc. of 95 per cent ethanol containing 5 cc. of concentrated HCl. The precipitate, collected by centrifugation, was washed with 125 cc. of 66 per cent ethanol and redissolved in 250 cc. of water and 16 cc. of 2 N ammonia. The precipitate obtained from the filtered solution with 500 cc. of alcohol and 4 cc. of concentrated HCl was suspended in 25 cc. of water and subjected to dialysis against running water (14 hours) and several changes of ice-cold distilled water (20 hours). The ribonucleic acid was collected, washed with 66, 98, and 100 per cent ethanol and with ether, and dried *in vacuo* over P_2O_5; it weighed 7.2 gm. For analysis, the substance was dried in a high vacuum at 60° for 3 hours and weighed out under exclusion of moisture.

Found, N (Dumas) 15.3, P (Pregl-Lieb) 8.0

This nucleic acid showed the characteristic absorption spectrum in the ultraviolet (pH 6.5). For the maximum at 257.5 mμ an ϵ(P) value of 9800 was found; at the minimum of 229 mμ ϵ(P) was 4200 (for definitions, see (9)).

Pentose Nucleic Acid of Pig Pancreas[1]—The preparation of this material followed in all essentials the procedures described by Jorpes (10, 11) and by Levene and Jorpes (12). The free pentose nucleic acid gave no protein tests and contained only a small amount (3 per cent) of desoxypentose nucleic acid (diphenylamine reaction). The substance was, for analysis, dried as described above.

Found, N (Dumas) 15.4, P (Pregl-Lieb) 7.9

The absorption maximum of this preparation (pH 6.3) was at 256 mμ with an ϵ(P) of 9800; the corresponding value for the minimum at 228 mμ was 4200.

Ribose Nucleotides—Adenylic acid was prepared from commercial yeast nucleic acid (Schwarz Laboratories, Inc., New York) in the crystalline state by the recent method of Buell (13). A few modifications were introduced, which included the use of 0.5 N alkali for 24 hours at 37° for the cleavage of the nucleic acid, and the repeated precipitation of the nucleotide as the lead salt before the recrystallization of the free adenylic acid from water. The air-dried white crystalline substance contained N (Dumas) 18.54, P (Pregl-Lieb) 8.45 per cent. It lost 2.6 per cent of its weight when dried for 2 hours at 109° *in vacuo*.

Basic sodium guanylate was prepared as a white crystalline powder from

[1] We are indebted to Dr. F. Misani for help with this preparation.

the potassium acetate complex of guanylic acid, collected in the course of the preparation of adenylic acid, by the method of Steudel and Peiser (14). The material, dried at 77° *in vacuo*, contained N (Dumas) 13.27, P (Pregl-Lieb) 6.16 per cent.

Cytidylic acid (Preparation 1) was prepared from the acid hydrolysate of commercial yeast nucleic acid by the fractional crystallization of the brucine salts according to Levene (15, 16). The nucleotide, recrystallized from 35 per cent ethanol and dried *in vacuo* at 65°, contained N 12.3, P 9.0 per cent, and had a rotation of $[\alpha]_D^{27} = +50.3°$ (0.1 per cent solution in water). Another specimen of cytidylic acid (Preparation 2) was obtained through the courtesy of Dr. S. J. Thannhauser and Dr. G. Schmidt. It contained, after recrystallization from 35 per cent ethanol, N 12.4, P 9.5 per cent, and had $[\alpha]_D^{27} = +50.8°$ (0.1 per cent solution in water). Levene (16) found for this nucleotide a rotation of $[\alpha]_D^{50} = +48.5°$.

Quantitative Estimation of Purines in Nucleic Acids

The nucleic acid was dried in a high vacuum at 60° for 3 hours and 5 to 8 mg. of the preparation, weighed on a micro balance, were placed in a small Pyrex bomb tube (160 × 5 mm.); 0.5 cc. of N sulfuric acid was added and the sealed tube heated for 1 hour at 100° in a boiling water bath. The clear solution was allowed to cool and was transferred, by means of a long capillary pipette, into a 1 cc. volumetric flask. The tube walls were rinsed three or more times with a few drops of 0.1 N H_2SO_4. The washings served to bring the hydrolysate up to volume. When the solvent system used for the subsequent purine separation consisted of *n*-butanol, morpholine, diethylene glycol, and water (7), the undiluted hydrolysate in the volumetric flask was first adjusted to pH 0.8 to 1 by means of a few drops of 30 per cent aqueous NaOH and then brought to a volume of exactly 1 cc. with 0.1 N H_2SO_4, the washings being used for this purpose. The pH adjustment was controlled by dipping the tip of a sliver of hydrion paper (pH 0 to 1.5) into the solution. When morpholine was omitted from the solvent mixture (7), no alkali was added and the solution was brought up to volume directly with 0.1 N H_2SO_4.

Several 0.01 to 0.02 cc. portions of this solution, dispensed on paper, as described in the preceding publication (7), by means of an accurate micrometric burette, served for parallel separations and estimations. As a rule, six determinations were carried out simultaneously with the same hydrolysis fluid. The procedures used for the development of the guide strip, which indicated the number and positions of the separated purines, and for the extraction and the identification and estimation of the components by spectroscopy in the ultraviolet followed exactly the methods described

before (7).[2] In all analyses, a drop of the hydrolysate and a drop of a purine test mixture of known composition were chromatographed side by side on a separate paper sheet, in order to compare the positions of the adsorbates on the chromatogram and thereby to verify the identification of the separated components.

Quantitative Estimation of Pyrimidines in Nucleic Acids

A portion, weighing between 15 and 25 mg., of the nucleic acid, that had been dried in a high vacuum at 60° for 3 hours, was weighed exactly into the special vessel shown as *C* in Fig. 1. Absolute methanol (0.5 to 1 cc.) was added and dry HCl gas passed, by means of a capillary, through the suspension with complete exclusion of moisture. A copper spiral through which cold water circulated converted the neck of the flask into a reflux condenser. (The experimental arrangement is illustrated in Fig. 1.) Within about 30 minutes the nucleic acid dissolved in the methyl alcohol, which became warm, and the purine hydrochlorides began to precipitate. The passage of HCl gas then was continued for 3 to 5 hours while the mixture was kept at 50°. After being chilled overnight with rigorous exclusion of moisture, the closed reaction vessel was centrifuged. The supernatant was quantitatively transferred to a bomb tube (220 mm. long, inside diameter 65 mm., outside diameter 85 mm.) by means of a capillary siphon which was actuated by suction.[3]

The yellowish methanol solution was evaporated at about 45° under a nitrogen current and the evaporation was continued, with the addition of small portions of fresh methanol, until the alcohol vapors carried almost no acid. About fifteen evaporations were, as a rule, necessary. The residue was dried overnight *in vacuo* over $CaCl_2$ and KOH. After the introduction of 0.5 cc. of concentrated formic acid (98 to 100 per cent, Eastman Kodak), the bomb tube was sealed and heated at 175° for 2 hours. It then

[2] It might be pointed out that it is possible to ascertain the presence on the chromatogram of minor purine or pyrimidine components whose concentrations are insufficient to permit their direct demonstration as Hg salts. As the relative positions of the individual purines and pyrimidines on the chromatogram are known (7), the extract of a segment, removed from the paper strip at the location of the suspected substance, may serve for the spectroscopic examination. In this manner, the pentose nucleic acid of pancreas was tested for thymine, but none was found.

[3] It was found preferable to omit the washing of the purine hydrochloride sediment, since this resulted invariably in the contamination of the pyrimidine fraction by purines, even when cold methanol saturated with gaseous HCl served as the wash fluid. The precipitated purine hydrochlorides, dissolved in 0.1 N H_2SO_4 and brought up to a known volume, may be subjected to separation by chromatography and estimation. The results, however, were not as constant as when the purine hydrolysis was carried out with N H_2SO_4, as described in the preceding section.

was chilled to 0° and, because of inside pressure, opened with all necessary precautions.

To the dark brown hydrolysate 2 to 3 drops of about 40 per cent NaOH solution were added, which effected the flocculation of the pigment and the clarification of the solution.[4] The tube was centrifuged, the light yellow supernatant transferred to a 1 cc. volumetric flask, and the centrifugation

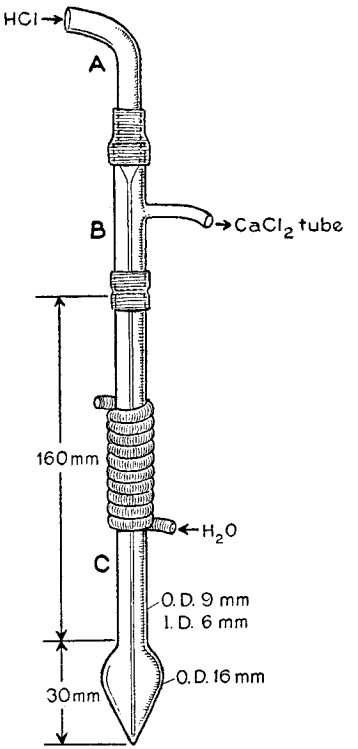

FIG. 1. Arrangement for the hydrolysis of very small amounts of nucleic acid The capillary tube *A* is connected through the adapter *B* with the reaction vessel *C*.

residue washed, at least three times, with 0.1 to 0.2 cc. portions of warm water. The centrifuged washings were used to bring the volume of the hydrolysate up to 1 cc. Several, usually six, exactly measured 0.01 to 0.02 cc. portions of this solution were deposited on the paper sheets serving for the chromatographic separation and were neutralized with gaseous ammonia. The separation with *n*-butanol-water as the solvent, the development, the extraction, and the identification and quantitative determination

[4] When insufficiently clarified brown solutions are subjected to chromatography, dark tongues form on the paper which interfere with the quantitative spectroscopy.

of the separated pyrimidines were carried out by the method previously described (7).

Control Experiments

Resistance of Pyrimidines to Acid Treatment—It is well known that the liberation of pyrimidines from nucleic acids requires an extremely drastic treatment, *e.g.* autoclaving at 175° for several hours with 20 per cent HCl (17) or 25 per cent H_2SO_4 (18). It appeared of interest to take advantage of the ease with which changes in the composition of pyrimidine mixtures can be followed with the new chromatographic method (7) by studying the effect of various acids on a mixture of uracil, cytosine, and thymine.

TABLE I

Resistance of Pyrimidines to Treatment with Strong Acid

A mixture of pyrimidines of known concentration was dissolved in the acids indicated below and heated at 175° in a bomb tube. The concentration shifts of the individual pyrimidines were determined through a comparison of the recoveries of separated pyrimidines before and after the heating of the mixture.

Experiment No.	Acid	Heating time	Concentration shift, per cent of starting concentration		
			Uracil	Cytosine	Thymine
		min.			
1	HCl (10%)	90	+62	−63	+3
2	10 N HCOOH + N HCl (1:1)	60	+3	−5	0
3	10 N HCOOH + N HCl (1:1)	120	+24	−19	0
4	HCOOH (98 to 100 %)	60	0	−1	−2
5	HCOOH (98 to 100 %)	120	0	+2	+1

The acids examined were 10 per cent hydrochloric acid, a mixture of equal parts of 10 N formic and N hydrochloric acids (19), and, finally, pure formic acid (98 to 100 per cent).

The original solution that served for the experiments contained 49.1 mg. of uracil, 50.4 mg. of cytosine, and 44.4 mg. of thymine in 10 cc. of 10 per cent HCl. A 2 cc. aliquot of this solution was neutralized with concentrated aqueous NaOH and diluted with water to an exact volume of 5 cc. Several 0.01 cc. portions of this neutral solution were subjected to chromatographic separation on filter paper with *n*-butanol-water as the solvent and to quantitative spectroscopic examination of the separated components (7). Another 2 cc. aliquot of the original test mixture was heated in a bomb tube for 90 minutes at 175°, neutralized and diluted to 5 cc., as described before, and likewise subjected to quantitative separation. The experiments with other acids were carried out in a similar manner. The results, assembled in Table I, indicate the instability of cytosine,

which was to a large extent converted to uracil, in acids other than formic acid; uracil and thymine resisted the acid treatment.

Hydrolysis of Yeast Ribonucleic Acid with Strong HCl—Following the liberation of purines by hydrolysis with gaseous HCl in dry methanol and the concentration of the supernatant from the purine hydrochlorides to dryness, as described above, the pyrimidine nucleotide residue was heated, in a sealed tube, with 0.3 cc. of 10 per cent hydrochloric acid at 175° for 2 hours. The pyrimidines were separated in the usual manner. Under these conditions the nucleic acid yielded 2.1 per cent of cytosine, 7.0 per cent of uracil. A comparison with the quantitative estimations summarized in Table VI will show that the hydrolysis with strong mineral acid produced an enormous shift in the relative proportions of the two pyrimidines, although the total amount recovered was nearly the same. In the HCl hydrolysate the mole proportions of pyrimidine to phosphoric acid were cytosine 0.073, uracil 0.242; the cytosine N amounted to only 5.2, the uracil N to 11.4 per cent of the nucleic acid N; the molar cytosine to uracil ratio was 0.3.

Hydrolysis of Yeast Ribonucleic Acid with Formic and Hydrochloric Acids—The following experiments were designed to exclude the possibility that the attacks on the nucleic acid by formic and by hydrochloric acids were directed against different groupings and were selective with respect to the proportions of liberated pyrimidines. From 48.052 mg. of yeast ribonucleic acid (dried in a high vacuum) the purines were split off as the hydrochlorides, in the manner described, by means of methanolic HCl. The methanolic supernatant was evaporated and freed of HCl and the residue transferred to a 2.5 cc. volumetric flask and dissolved in methanol. Two 1 cc. portions of this solution, designated Solutions A and B, were subjected to hydrolysis. Solution A was taken to dryness, the residue heated with 1 cc. of concentrated formic acid for 2 hours at 175° in a sealed bomb tube, and the pyrimidine distribution determined in the usual manner. The evaporation residue of Solution B was similarly treated with formic acid. The hydrolysate was evaporated (at the end with frequent additions of absolute ethanol) under nitrogen at 45° and the residue again subjected to hydrolysis in a sealed tube, this time with 1 cc. of 20 per cent HCl, at 175° for 2 hours. The following results on the pyrimidine distribution again confirmed the destructive effect of mineral acids on cytosine, but ruled out the assumption of a differential action of formic and hydrochloric acids: Solution A, cytosine 6.8, uracil 2.4, total pyrimidines 9.2 per cent; Solution B, 3.5, 5.7, and 9.2 per cent, respectively.

Hydrolysis of Nucleotides—3.264 mg. of *adenylic acid* were hydrolyzed with N sulfuric acid at 100° for 1 hour as described above. Aliquots corresponding to 32.64 γ of the nucleotide yielded, when chromatographed in

n-butanol-diethylene glycol-water, 11.1 γ of adenine (95 per cent of the amount expected from the N content). *Sodium guanylate*, similarly treated, gave, per 29.08 γ of the nucleotide, 8.34 γ of guanine (100 per cent).

The hydrolysis of *cytidylic acid* was studied with the two preparations mentioned before. With Preparation 1 the liberation of phosphoric acid and of cytosine by treatment with concentrated formic acid for various periods was followed and compared with the behavior of yeast ribonucleic acid under analogous conditions. Both the nucleotide and the nucleic acid were subjected to the operations necessary for the removal of the purines, the hydrolysis with formic acid at 175° in a bomb tube, and the determination of the pyrimidines in the manner described. At the same

TABLE II

Liberation of Pyrimidines and of Phosphorus from Yeast Ribonucleic Acid and from Cytidylic Acid by Heating with Concentrated Formic Acid

Duration of heating at 175°	Yeast ribonucleic acid		Cytidylic acid		
	Inorganic P	Total pyrimidines (cytosine + uracil)	Inorganic P	Cytosine	Cytosine N
min.	*per cent of total P*	*per cent*	*per cent of total P*	*per cent*	*per cent of nucleotide N*
0	6		0		
30	83				
60	85		76		
90	100	8.2	100	22.7	70
120	100	9.2	100	25.8	80
150				25.6	79
210		9.1			
300		9.2			

time, the total and the inorganic phosphorus contents were estimated colorimetrically in dilutions of the hydrolysates. The results will be found in Table II. Another set of experiments with cytidylic acid (Preparation 2), reproduced in Table III, was designed to compare the effects of 90 and 99 per cent formic acids and of very strong hydrochloric acid. All hydrolyses were performed in the customary manner in bomb tubes at 175°. It will be seen (Experiment 1a) that the action of fresh acid on a hydrolysate produced little additional effect. The behavior of cytidylic acid preparations toward hydrolysis obviously will require additional study. A glance at Tables II and III will show that the recovery of cytosine amounted in no case to more than about 80 per cent of the nucleotide nitrogen. It was even lower with strong hydrochloric acid as the hydrolyzing agent when, as expected, a portion of the cytosine was converted to uracil (Experiment

3 in Table III). The reasons for this refractoriness of cytidylic acid are not yet clear.

Composition of Yeast Ribonucleic Acid

Purines—The only purines encountered on the chromatograms were adenine and guanine. The absorption maximum of adenine (in 0.1 N HCl) was at 263 mμ, that of guanine (in N HCl) at 249 mμ. A series of estimations, each representing the average of at least six parallel determinations, is assembled in Table IV. The average of all adenine values reported is 9.1 per cent, that of all guanine values 10.2 per cent. If the value for guanine found for Hydrolysate 13 (Table IV), which appears rather out of range, is disregarded, the guanine average becomes 10.0 per

TABLE III

Hydrolysis of Cytidylic Acid

Experiment No.	Acid	Duration of heating at 175°	Pyrimidines found			
			Cytosine	Cytosine N	Uracil	Uracil N
		hrs.	*per cent*	*per cent of nucleotide N*	*per cent*	*per cent of nucleotide N*
1	99% HCOOH	2	23.8	73	0	0
1a*	99% "	4	25.4	78	0	0
2	90% "	2	26.0	79	0	0
3	20% HCl	2	16.6	51	6.5	13

* An aliquot of the hydrolysate obtained in Experiment 1 was evaporated to dryness under N_2 at 45° and the residue heated with a fresh portion of concentrated formic acid for 2 hours. Only traces of absorbing material were found at the chromatographic position of uracil.

cent. The experiment with Hydrolysate 22 is for several reasons considered as the most reliable; it will be seen that the figures reported for it differ only very slightly from the averages computed for the entire series. When adenine in Hydrolysate 4 and guanine in Hydrolysate 22 each were determined with the use of two different solvent systems, the results were in very good agreement.

Pyrimidines—Cytosine and uracil, the latter a relatively minor component, were the pyrimidines found in the hydrolysates. The absorption maximum for cytosine was at 268 mμ, that for uracil at 259 mμ (solutions in water). The results of several determinations are summarized in Table V. The average value for cytosine was 6.7, that for uracil 2.3 per cent. These figures, it is felt, require an upward correction of 5 per cent. As was pointed out above (see also foot-note 3), it was found advantageous to omit the washing of the purine hydrochlorides that precipitated in the course of the hydrolysis with methanolic HCl, in order to avoid the contamination of

the pyrimidine fraction. This involved, of course, a small loss in pyrimidines owing to the retention of some pyrimidine nucleotide by the purine sediment. For an estimate of the extent of this loss, hydrolysates of thymus nucleic acid were employed, since the position of thymine on the chromatogram rendered it the pyrimidine least affected by contamination with purines (7). It was thus found, in comparative hydrolysis experiments, that approximately 5 per cent of the thymine was retained in the

TABLE IV

*Purine Content of Yeast Ribonucleic Acid**

Hydrolysate No.	Solvent system	Adenine	Guanine
		per cent	*per cent*
1	*n*-Butanol-morpholine†	9.7	
2	"	8.6	
4	"	9.5	
4	*n*-Butanol-diethylene glycol-morpholine	9.5	
7	" "	9.2	
10	" "	9.9	
13	" "	9.6	12.2
14	" "	8.6	9.7
21	" "	8.1	9.3
22	" "	9.0	10.1, 10.4‡
24	" "		10.3
25	" "		10.0
27	" "	8.4	9.9

* Each value represents the average of at least six parallel determinations (concordant within a range of 5 per cent) on the same hydrolysate.

† This solvent system, described in the preceding paper (7) for qualitative separations, may be used for the estimation of adenine but not of guanine. The procedures are the same as for the solvent containing diethylene glycol.

‡ In this determination *n*-butanol-diethylene glycol (in NH_3 atmosphere) was employed as the solvent.

purine hydrochloride precipitate. For this reason, the pyrimidine figures given in Tables VI and VIII, which summarize the distribution of the nitrogenous components of pentose nucleic acids, were corrected by this factor.

Proportions and Balances—Table VI provides a survey of the distribution of purines and pyrimidines in the yeast ribonucleic acid. The fact that the purines or pyrimidines liberated by the hydrolysis of a nucleotide contain 1 hydrogen atom more than the corresponding radicals present in the uncleaved compound was left out of consideration, since the contributions from this correction would have been negligible. It will be seen that the molar proportions (taking uracil as 1) and the mole per mole of phosphorus

ratios of the nitrogenous constituents rule out the existence of a regular tetranucleotide. It is of course understood that the computations presented here are not at all dependent upon any particular assumption regarding the structure of the nucleic acid analyzed. That the recoveries in terms of total nitrogen and of total phosphorus were closely similar, but

TABLE V

*Pyrimidine Content of Yeast Ribonucleic Acid**

Hydrolysate No.	Cytosine	Uracil
	per cent	*per cent*
32	†	2.1
34	7.4	2.3
35	6.5	2.4
36	6.1	2.3
38	6.8	2.4

* Each value represents the average of at least six parallel determinations (concordant within 5 per cent) on the same hydrolysate. In all separations *n*-butanol (saturated with water) served as the solvent.

† The absorption spectrum of the cytosine solution, isolated in this experiment, indicated contamination.

TABLE VI

*Yeast Ribonucleic Acid; Proportions and Balances**

Compound	Content in nucleic acid	Nitrogen in nucleic acid	N accounted for	Purine N / Pyrimidine N	Mole per mole P	P accounted for	Moles per 4 moles P	Molar proportions
	per cent	*per cent*	*per cent of nucleic acid N*			*per cent of nucleic acid P*		
Adenine	9.1	4.72	30.9		0.261	26.1	1.04	3.2
Guanine	10.0	4.63	30.3		0.256	25.6	1.02	3.1
Cytosine	7.0	2.65	17.3		0.244	24.4	0.98	3.0
Uracil	2.4	0.60	3.9		0.083	8.3	0.33	1.0
Total nucleic acid			82.4	2.9		84.4		

* The nucleic acid preparation contained N 15.3, P 8.0 per cent.

did not quite reach 100 per cent, may be significant, as will be pointed out later.

Composition of Pancreas Pentose Nucleic Acid

As was the case with the yeast nucleic acid discussed immediately above, adenine, guanine, cytosine, and uracil were the four nitrogenous constituents encountered on the chromatograms. Tests carried out with extracts

collected in the region of thymine adsorption (compare foot-note 2) failed to indicate the presence of this pyrimidine. The series of estimations reproduced in Table VII revealed an average content of adenine 5.7, guanine 15.5, cytosine 5.5, uracil 1.2 per cent. Guanine, therefore, was by

TABLE VII

*Purine and Pyrimidine Content of Pancreas Pentose Nucleic Acid**

Hydrolysate No.	Adenine	Guanine	Cytosine	Uracil
	per cent	*per cent*	*per cent*	*per cent*
1	6.0	15.4		
2†	5.5	16.0		
3	5.6	15.2		
5			5.3	1.5
6			5.9	1.1
7			5.2	1.0

* Each value represents the average of at least six parallel determinations (concordant within 5 per cent) on the same hydrolysate. The purine determinations were all carried out with *n*-butanol-diethylene glycol-morpholine as the solvent; for the estimation of pyrimidines *n*-butanol was employed.

† In this experiment the duration of hydrolysis was doubled to 2 hours.

TABLE VIII

*Pancreas Pentose Nucleic Acid; Proportions and Balances**

Compound	Content in nucleic acid	Nitrogen in nucleic acid	N accounted for	Purine N / Pyrimidine N	Mole per mole P	P accounted for	Molar proportions
	per cent	*per cent*	*per cent of nucleic acid N*			*per cent of nucleic acid P*	
Adenine	5.7	2.95	19.2		0.166	16.6	3.6
Guanine	15.5	7.18	46.6		0.402	40.2	8.8
Cytosine	5.8	2.19	14.2		0.205	20.5	4.5
Uracil	1.3	0.32	2.1		0.046	4.6	1.0
Total nucleic acid			82.1	4.0		81.9	

* The nucleic acid preparation contained N 15.4, P 7.9 per cent.

far the major component of this nucleic acid. The molar guanine to adenine ratio was 2.4. It might be mentioned that in the analysis of a second less pure preparation of a pancreas nucleic acid which is not discussed in detail here, since it contained some protein and about 6 per cent of desoxypentose nucleic acid, the following figures were found: adenine 4.1, guanine 16.5 per cent.

The proportions and balances, computed as for yeast ribonucleic acid, are tabulated in Table VIII.

Sugar Component of Pentose Nucleic Acids

Preliminary experiments were carried out on the nature of the pentose liberated by the cleavage of the purine nucleotide moiety of the nucleic acids studied. It was of interest to ascertain the possibility of examining the sugars in minute amounts of hydrolysates by means of filter paper chromatography (20). This proved feasible, particularly when the development method for sugars recently described from this laboratory (21) was employed.

The paper used was Schleicher and Schüll, No. 597. The solvent system consisted of a mixture of 4 volumes of *n*-butanol, 1 volume of ethanol, and 5 volumes of water; the upper organic layer was used for the chromatographic separation which was permitted to proceed for about 12 hours at room temperature. In all experiments, adjacent chromatograms were made with authentic D-ribose and also with D-xylose which is the pentose nearest to ribose in partition behavior.

3 mg. portions of the yeast and pancreas nucleic acids each were hydrolyzed with 0.3 cc. of N H_2SO_4 at 100° for 1 hour. During this time the volume was kept approximately constant by the addition of a few drops of water. For the chromatograms 0.01 cc. portions of the hydrolysates were employed; the drops were neutralized on the paper by exposure to gaseous NH_3. Following the separation the paper strips were treated with *m*-phenylenediamine dihydrochloride as described before (21). The strongly fluorescent spots indicated the presence of one sugar only, which was in all probability D-*ribose*. The R_F values of the sugar components of both yeast and pancreas nucleic acids were 0.30 and coincided completely with the position of authentic ribose. D-Xylose, on the other hand, occupied an unmistakably different position, with an R_F value of 0.24.

Appendix: Characterization of All Nitrogenous Constituents in One Nucleic Acid Sample

In cases in which the scarcity of the available material precludes two separate weighings for the determination of the purines and the pyrimidines respectively, the following procedure may be employed, which involves the liberation of the purines by mild acid hydrolysis and the, at least partial, precipitation of the pyrimidine nucleotides by uranyl acetate in a manner similar to that followed with the purine nucleotides (compare (22)). With regard to the estimation of the purines the procedures are identical with the ones described above, and the figures found have the same significance; the figures for the pyrimidines, however, have at best a semiquantitative value, though the method may be of interest for the characterization of the pyrimidines in very small amounts of nucleic acid.

Between 15 and 25 mg. of the nucleic acid preparation, previously dried in a high vacuum at 60° for 3 hours, were weighed exactly into a small bomb tube, 1.5 cc. of N H_2SO_4 were added, and the sealed tube was heated for 1 hour at 100°. The hydrolysate was brought up to a volume of exactly 2.5 cc. with 0.1 N H_2SO_4, either directly or after adjustment to pH 0.8 to 1, as was described above in the section on the estimation of purines. A portion of this solution (0.3 cc.) served for the purine determinations.

An exact 2.0 cc. aliquot of the solution was placed in a centrifuge cup and adjusted to pH 7 with the help of a mixed indicator. Guanine, which precipitated in part, was centrifuged off and washed once with water. From the united supernatants the nucleotides were precipitated by the addition of a sufficient amount of a saturated (7 per cent) uranyl acetate solution. 10 minutes later the mixture was centrifuged and the supernatant brought to neutrality, at which time a second precipitation occurred. The supernatant was discarded and the united precipitates were dissolved in about 1 cc. of 2 N HCl in order to remove purine traces. The neutralization of this acidic solution brought about the reprecipitation of the uranium salts. The mixture was treated with 1 to 2 drops of the uranyl acetate solution, allowed to stand for 10 minutes, and then centrifuged. The sediment was dissolved or suspended in 0.3 cc. of about 100 per cent formic acid[5] and the solution transferred quantitatively to a bomb tube, two 0.1 cc. portions of formic acid being used for washing. The sealed bomb tube was heated to 175° for 2 hours; the cooled hydrolysis mixture was freed of a white sediment by centrifugation and the supernatant introduced into a 1 cc. volumetric flask. The sediment was washed as often as possible with small amounts of water which served to bring the volume to 1 cc. The chromatography and estimation of the pyrimidines then were carried out in the prescribed fashion.

The total of pyrimidines recovered in this manner was considerably lower than in the procedure discussed above for the quantitative estimation of pyrimidines. The loss was largely attributable to a disappearance of cytosine which in this case, however, was not accompanied by a rise in uracil. The following pyrimidine figures were, for instance, found for yeast ribonucleic acid: cytosine 4.3 and uracil 1.8 per cent, which corresponded to 64 and 78 per cent respectively of the authentic values given in Table V. For the pancreas nucleic acid the figures were cytosine 2.9 and uracil 1.0 per cent, amounting to a recovery of 53 per cent of the cytosine and of 83 per cent of the uracil reported in Table VII.

That this loss in cytosine could not be due to the partial solubility of the

[5] While the uranium precipitate of cytidylic acid was completely soluble at this state, a small amount of an insoluble residue remained when hydrolysates of pentose nucleic acids were examined.

uranium salt of cytidylic acid itself was shown in a control experiment with cytidylic acid (Preparation 1). 99.8 mg. of the nucleotide were dissolved in 0.1 N HCl to give a volume of exactly 25 cc. It was found that the entire P contained in 1 cc. aliquots of this solution went into the precipitate produced by uranyl acetate. However, the uranium precipitates thus obtained, when heated at 175° with concentrated formic acid for varying periods, yielded cytosine values that were considerably lower than those given in Table II. The percentages of cytosine found were as follows: after 90 minutes heating 21.4, after 120 minutes 22.7, after 150 minutes 18.4.

DISCUSSION

Since this study is in many ways the first of its kind, the discussion may well begin with a brief consideration of its limitations and shortcomings. First of all, it deals solely with the *composition* of nucleic acids; its bearing on the problem of nucleic acid structure is only indirect. Although the recognition of structural principles requires an exact knowledge of the nature and the proportions of all constituents, the findings reported here demand no particular assumption with respect to a specific arrangement of the components or to the type of linkage holding them together. Secondly, it must be understood that all figures presented refer to the hydrolysates only. Groupings that are not cleaved in the course of hydrolysis and compounds that are destroyed during the cleavage of the nucleic acid or that are not liberated at all will, of course, escape detection. The hydrolysis may, on the other hand, lead to the production of artifacts, not present as such in the starting material. These reservations naturally apply to the constituent analysis of all complicated organic substances.

The quality of the nucleic acid preparations also may be responsible for a number of errors. It is known that pentose nucleoproteins in general require a much more drastic treatment for the detachment of the nucleic acid than is the case with the corresponding desoxypentose nucleic acid complexes; and the commercially available preparations of yeast ribonucleic acid probably are badly degraded, as indicated by the numerous purification procedures found in the literature (6) and also by the not infrequently discordant results obtained with different specimens (2). The situation is more favorable with respect to the pentose nucleic acid from pancreas and even more so as regards the desoxypentose nucleic acids which will be discussed in a forthcoming publication. In any event, general principles of composition can already be recognized in the pentose nucleic acids.

That satisfactory methods of hydrolysis are among the most important requisites for the complete characterization of the composition of nucleic acids requires no added emphasis. The liberation of the purines is generally

assumed to be achieved by mild acid hydrolysis (compare p. 223 in (1) and also (19)); and this was definitely proved in the present study with purified adenylic and guanylic acids from which adenine and guanine in the respective yields of 95 and 100 per cent of the expected amounts were isolated. The complete release of the pyrimidines presents a much more difficult problem. The method commonly employed, *viz.* prolonged autoclaving with strong mineral acid at a high temperature, must, in the light of the findings reported here, have led to very erroneous conclusions. It has now been found that under these conditions a large part of the cytosine of the nucleic acid is converted to uracil. It was, therefore, necessary to develop a hydrolysis procedure that would permit the attainment of the maximal liberation of constituents without the production of artifacts or of a shift in proportions. Concentrated formic acid was finally chosen for this purpose. This treatment yielded values that were often higher, but in no case lower, than those for total pyrimidine content found with strong mineral acid. Furthermore, since it effected no appreciable conversion of cytosine to uracil, the important question of the presence of uracil as such in the pentose nucleic acids became susceptible of an answer.

The great advantage of the estimation method employed here lies in the fact that it makes possible a complete survey of the distribution of purines and pyrimidines in minute amounts of nucleic acid hydrolysates[6] and that it is capable of distinguishing between the different nitrogenous constituents. Since the total hydrolysate is subjected to a partition between solvents, all components, regardless of their physical properties, have an equal chance of being demonstrated without having to undergo wasteful conversions into derivatives.

The accuracy of the chromatographic separation of the purines and pyrimidines has been discussed in the preceding communication (7). It now remains to consider the faults that could vitiate the analytical results on nucleic acids. If pyrimidines had partly been liberated in the course of the mild hydrolysis employed for the release of purines, slightly too high a value could have resulted for adenine, due to contamination with uracil and, perhaps, also with cytosine. This is, however, unlikely, not only because of the spectroscopic controls that were invariably applied, but also because of the well known stability of the pyrimidine nucleotides. The latter property could, however, be a cause of error in the pyrimidine determinations; this will be taken up later. Another error could have been

[6] Even smaller quantities of nucleic acid than were used here could doubtless be employed for the estimations, if the hydrolysates were adjusted to a lesser volume, or if, without volume adjustment, a known amount of a substance were added to the hydrolysates which, serving as a marker, could be separated and estimated quantitatively on the chromatogram.

introduced by the contamination of the pyrimidine fraction with purines that had not been removed completely as the hydrochlorides. This is even less likely; in the chromatographic procedure used, the cytosine fraction could have contained traces of hypoxanthine which, however, probably is not a common nucleic acid constituent and is quite different spectroscopically from cytosine. The absorption maxima of uracil and of adenine, its possible contaminant, are nearer, but since no uracil spot was detected in the corresponding hydrolysates of desoxypentose nucleic acids, contamination with adenine is not likely to have played an important rôle.

Before the results presented in this study are compared with those of previous workers, one additional point should be stressed. The inspection of Tables VI and VIII will show that in both nucleic acid preparations a certain proportion (15 to 18 per cent) was not accounted for. Several reasons could be responsible for this deficit. It could, for instance, have resulted from the summation of hydrolysis losses affecting equally the estimations of all the purines and pyrimidines. One observation would seem to favor this assumption. When the total number of gm. atoms of nitrogen found in the hydrolysates is divided by the total number of moles of the four bases in which they are contained (see molar proportions in Tables VI and VIII), the quotients, which indicate the average nitrogen content of each nitrogenous constituent, are in very good agreement with the atomic nitrogen to phosphorus ratio calculated from the analytical values found for the intact nucleic acids. For yeast ribonucleic acid this quotient is 4.1, the atomic N:P ratio 4.2; the corresponding figures for the pancreas nucleic acid are 4.3 and 4.3. This agreement suggests that the hydrolyses did not result in a considerable fractionation.

Another possibility is that the nucleic acids contained small amounts of unidentified components which either were resistant to hydrolysis or gave rise to substances that could not be demonstrated by the chromatographic methods. In this connection, attention may be drawn to the behavior of isolated cytidylic acid toward hydrolysis, discussed above in conjunction with the experimental findings. Several peculiarities exhibited by ribonucleic acids, *e.g.* the lability of the internucleotide linkages toward alkali, cannot be explained on the basis of the currently assigned structures; and these problems will have to be investigated further in connection with the behavior of isolated pyrimidine nucleosides and nucleotides. It will be remembered that dephosphorylation of the nucleic acids appeared complete under the conditions of hydrolysis (Table II); but it is not impossible that a small proportion of differently linked pyrimidines was not liberated by acid hydrolysis. Chromatographic separation studies on the distribution of the constituent nucleotides and other investigations, which are being carried out at present, will perhaps contribute to a decision.

The identification of the sugar component that is associated with the purine moiety of the pentose nucleic acids studied here was attempted, in order to test the possibility of applying a chromatographic microprocedure to the investigation of the carbohydrates present in minute amounts of nucleic acids. Only one sugar was detected in the hydrolysates of the yeast and the pancreas nucleic acids. It was identical in partition behavior with D-ribose, which is in accordance with the original findings of Levene and Jacobs (23).

When the tetranucleotide structure of yeast ribonucleic acid is discussed in the literature (compare, for instance, (3) p. 198), reference is usually made to a publication of Levene (24) that is said to provide the evidence for the occurrence in this nucleic acid of the two purines and the two pyrimidines in equimolecular proportions. Actually, this is far from correct. From 10 gm. of nucleic acid (N 15.2, P 8.6 per cent) 2.0 gm. of adenine picrate and 1.0 gm. of guanine were isolated. The same quantity of nucleic acid yielded cytosine, as what is described as 3.0 gm. of a crude picrate, and uracil whose isolation was reported without any indication of its weight. The nitrogenous bases were, therefore, distributed as follows: adenine 7.1 per cent (0.19 mole per mole of P); guanine, in good agreement with the present findings, 10 per cent (0.24 mole); cytosine (if the crude derivative is considered as cytosine picrate) 9.8 per cent (0.32 mole); no value can be assigned to uracil. The corresponding molar proportions, *viz.* adenine 1.0, guanine 1.3, cytosine (?) 1.7, hardly lend themselves to the formulation of a regular tetranucleotide. The figures assembled in Table VI in the present paper provide, in fact, much better evidence of regularity, but further considerations of this kind should be postponed.

Most of the other evidence is of a more circumstantial nature, *e.g.* the rates of liberation of phosphoric acid (25, 26) and of sugar (26, 27), the calorimetric behavior of yeast nucleic acid (28), the amount of total purine nitrogen liberated (19), etc. A discussion of the analytical data submitted in a preliminary form by Loring *et al.* (29) should, in view of a recent note (30), await the presentation of experimental details.

The pentose nucleic acid of pancreas, while much investigated, has not had as important a part as yeast nucleic acid in the development of the conception of nucleic acid structure, though its peculiar composition early served to draw attention to the far from simple problems involved. It was suspected of not fitting into the pattern of a regular tetranucleotide and was in turn formulated as a guanylic acid complex of ribonucleic acid and as a hexa- or pentanucleotide. Hammarsten (31) assumed a guanine to adenine ratio of 3:1, Steudel (32) of 4:1, Levene and Jorpes (12) found in different preparations 3.3 and 4.6 times as much guanine as adenine, and Jorpes (11) twice as much guanine as adenine. Since some of the guanylic acid present in this material appears to be bound rather feebly, it is probable

that different methods of isolation led to products of somewhat varying composition, quite apart from the uncertainties inherent in the estimation methods.

It will be seen that the findings summarized in Table VIII correspond to about 4 molecules of adenine and 5 of cytosine per 10 molecules of guanine. The ratio of purine to pyrimidine nitrogen was very high. Since pancreas is rich in ribonuclease which appears to act preferentially on the pyrimidine nucleotide portion of ribonucleic acids (33, 34), it is not impossible that some pyrimidine was liberated in the course of the preparation of the material.

One more point remains to be considered, namely the presence of uracil in the pentose nucleic acids. That cytosine is converted to uracil with relative ease has long been known (35); and in view of the extremely energetic methods commonly used for hydrolysis it is natural that the possibility of uracil being an artifact has formed the subject of lively controversies. Kowalevsky (36), in an investigation of yeast ribonucleic acid carried out in Steudel's laboratory, attempted to demonstrate the exclusive presence of three nitrogenous bases which she claimed to occur in the following molar proportions: adenine 1, guanine 1.6, cytosine 1.1. While the concentration of uracil in the hydrolysates of both pentose nucleic acids examined in the present study is very low, there is every reason to believe that it is a genuine constituent of the preparations. To what extent, however, the effect of deaminases and of other enzymes acting on the nucleic acids during their isolation may contribute to their final composition is a problem that will have to be considered separately.

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SUMMARY

The methods for the separation and estimation of adenine, guanine, cytosine, and uracil in minute amounts, described in the preceding communication, were applied to a study of the distribution of these nitrogenous constituents in hydrolysates of the ribonucleic acids of yeast and of pancreas. Since it was shown that under the customary conditions of hydrolysis with strong mineral acid cytosine was largely converted to uracil, concentrated formic acid was chosen as the hydrolyzing agent.

The presentation of detailed methods for the preparation of the hydrolysates and the estimation of the individual components is followed by a consideration of the proportions in which the purines and pyrimidines were found in the hydrolysates. A procedure, permitting the characterization of the nitrogenous constituents in very small quantities of nucleic acid,

and the application of chromatography on filter paper to the identification of the carbohydrate components of nucleic acids likewise are described.

The paper, which includes studies of the hydrolysis behavior of isolated nucleotides, concludes with a critical discussion of the findings.

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