A SPECTROPHOTOMETRIC PROCEDURE FOR THE ANALYSIS OF NUCLEIC ACID PURINES AND PYRIMIDINES, AND ITS APPLICATION TO FISH SPERM NUCLEIC ACIDS*

C. F. EMANUEL AND I. L. CHAIKOFF

Department of Physiology of the University of California School of Medicine, Berkeley, Calif. (U.S.A.)

I. INTRODUCTION

The quantitative analysis of nucleic acid purines and pyrimidines has been accomplished in several ways. In the most widely used procedure, the purine and pyrimidine components of the hydrolysed nucleic acid are first separated by paper chromatography, then eluted and measured quantitatively in a spectrophotometer[1]. In our hands, this method was found to be of low precision, necessitating many replicate analyses in order to obtain acceptable values. The poor precision resulted from impurities in the filter paper and from extensive manipulations. Furthermore, the recoveries of the heterocyclic bases rarely exceeded 80%, with losses the result of inadequately controlled hydrolysis of the nucleic acid.

A few reports have appeared on the use of starch-column chromatography[2] and ion-exchange resins for separating purines and pyrimidines[3] as well as nucleotides[4,5,6]. Although the recoveries are excellent, the methods are tedious and time-consuming, and require at least 50 h for the analysis of a single sample.

KERR *et al.*[7] and, later, LORING *et al.*[8] devised a procedure for analysis of 5–10 mg of ribonucleic acid (RNA) based upon differential spectrophotometry. In this method, the RNA was digested for 1 h, at 100°, with N H_2SO_4. The pH of the mixture was then adjusted to 1 and the purines were precipitated with silver nitrate solution. The silver-purine precipitate was filtered, washed, and decomposed to the free purines with hot hydrochloric acid. The purine-containing filtrate was then subjected to a two-component, differential spectrophotometric analysis by which the exact content of guanine and adenine was obtained. The pyrimidine nucleotide fraction of the hydrolysate (not precipitable with acidic silver solutions) was dephosphorylated with acid phosphatase, purified on an ion-exchange column, and also subjected to a two-component spectrophotometric analysis for cytidine and uridine content. Since 99% of the heterocyclic content of RNA was accounted for in terms of total nitrogen, this procedure has a decided advantage over paper-chromatographic methods in accuracy and probably in time.

In its present form, the LORING procedure for RNA is not applicable to deoxyribonucleic acid (DNA) analyses. In order to apply the principles of LORING's

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procedure to DNA, it was necessary to determine the optimum conditions for the perchloric acid hydrolysis of DNA. In addition, we determined the molar absorbancy coefficients of the pure purines and pyrimidines under the same experimental conditions as were used in our analyses. A method is described here for the hydrolysis and quantitative analysis of DNA which is precise and accurate. All quantitative manipulations, cooling, etc. of the silver-purine precipitates, and the enzymic hydrolysis and ion-exchange treatment used in the LORING procedure are eliminated. A complete duplicate analysis of at least a dozen samples can be carried out in one working day.

II. MATERIALS AND EQUIPMENT

The nucleic acid samples were isolated from salmon sperm* or hepatocarcinoma tissue** by the authors' procedures[9,10]. In a further effort to eliminate final traces of protein, we dissolved the highly purified nucleic acid samples in physiological saline (in which nucleoprotein is insoluble), and centrifuged the solutions at 100,000 × *g* for 60 min at 7°. The nucleic acid in the clear supernatants was precipitated with ethanol by standard procedure.

The purines and pyrimidines were isolated from salmon sperm DNA by the method devised for RNA by HUNTER AND HLYNKA[11]. Deoxyribonucleotides were purchased from the Biochemical Research Foundation, and recrystallized from warm water until suitable purity was obtained. Phosphorus and nitrogen analyses were done as previously described[10]. The analytical properties of the crystalline substances are given in Table I. The nitrogen and phosphorus contents of the nucleic acids (dried, *in vacuo*, at room temperature over $Mg(ClO_4)_2$) are given in Tables II and VII.

TABLE I

ELEMENTARY COMPOSITION OF NUCLEIC ACID DERIVATIVES

Compound	Calculated				Found			
	C	H	N	P	C	H	N	P
Adenine sulfate§ $(C_5H_5N_5)_2 \cdot H_2SO_4$	29.70	3.99			29.98	3.79		
Guanine hydrochloride $C_5H_5N_5O \cdot HCl$	31.99	3.20			31.89	3.34		
Thymine $C_5H_6N_2O_2$	47.61	4.79	22.21		47.63	4.93	21.82	
Cytosine $C_4H_5N_3O$	43.27	4.54	37.82		43.28	4.72	37.74	
Deoxyribocytidylic acid $C_9H_{14}N_3O_7P \cdot H_2O$			12.91	9.52			13.37	9.22, 9.30
Calcium thymidylate $C_{10}H_{14}N_2O_8P \cdot Ca \cdot 2H_2O$				7.80				7.72
Diammonium deoxyriboguanylate $C_{10}H_{14}N_5O_7P \cdot 2NH_4 \cdot 2H_2O$			23.39	7.38			22.95	7.65
Diammonium deoxyriboadenylate $C_{10}H_{14}N_5O_6P \cdot 2NH_4$			26.82	8.44			27.21	8.45

§ Purines and pyrimidines were dried over $Mg(ClO_4)_2$ and NaOH at 110° for 18 h, *in vacuo*. Nucleotides were dried at room temperature, *in vacuo*, over $Mg(ClO_4)_2$ and NaOH.

Silver perchlorate was prepared as follows: silver nitrate was converted to silver hydroxide with a stoichiometric amount of sodium hydroxide. After the silver hydroxide was thoroughly washed with water, it was mixed with a slight excess of 60% perchloric acid. The colorless solution was filtered through a sintered disk and then slowly concentrated over sulfuric acid, in the dark, until crystals formed. The silver perchlorate was recrystallized two or three times from a small

* Obtained from the Issaquah Fish Hatchery through the courtesy of the Washington State Department of Fish and Game.

** C_{954} hepatocarcinoma (obtained from Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine) was carried by serial transplantation in C_{57} leaden mice.

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TABLE II

ANALYTICAL PROPERTIES OF DNA SAMPLES

NaDNA source	*N* (%)	*P* (%)	*N/P*
Pink (Humpback) salmon sperm (*Oncorhynchus gorbuscha*)	15.61	8.55	1.83
King (Chinook) salmon sperm (*O. tshawytscha*)	14.78	8.15	1.81
Chum (Dog) salmon sperm (*O. keta*)	15.19	8.25	1.84
Sockeye (Red) salmon sperm (*O. nerka*)	15.10	8.25	1.83
Silver (Coho) salmon sperm (*O. kisutch*)	15.42	—	—

amount of hot water until a 20% solution of it had an absorbancy at 250 millimicrons of 0.25 or less.

The Beckman DU spectrophotometer was tested by standard procedures[12] for wavelength position and absorbancy intensity. Deviations in absorbancy (A_s) from National Bureau of Standards values were 1.2% (low) at 250 mμ, 0.6% (low) at 260 mμ, and 1.8% (low) at 280 mμ. The absorbancy values (Table III) used in this work were not corrected for these errors. Reproducibility of absorbancy readings and wavelength settings required the elimination of parallax errors in the manner described by GIBSON AND BALCOM[13]. This is particularly important for the procedure described here since the wavelength settings often fall on very steep portions of the absorption curves where a small wavelength error will result in a large absorbancy error.

The wavelength settings finally selected here are not the theoretically optimum ones. A compromise was necessary to avoid the shorter wavelength where interference from unknown foreign materials in nucleic acid hydrolysates was more likely to occur. The molar absorbancy indexes (a_M) obtained from the analytically pure purines and pyrimidines are given in Table III. The purine values deviate somewhat from those reported by LORING *et al.* The reasons for this are not known. However, equations derived from our absorbancy values gave calculated purine concentrations, much closer to known values for a standard mixture of purines than did the equations given by LORING *et al.*

TABLE III

MOLAR ABSORBANCY INDEXES (a_M) FOR DNA PURINES AND PYRIMIDINES

Compound	*Wavelength*		
	250 mμ	*262 mμ*	*280 mμ*
Thymine	5100	—	4030
Cytosine	2976	—	9600
Guanine	—	7842	6866
Adenine	—	12940	4608

III. GENERAL EQUATIONS FOR TWO-COMPONENT DIFFERENTIAL SPECTROPHOTOMETRY

It was our original intention to carry out a four-component differential analysis of DNA hydrolysates, and equations for such an analysis were developed. However, because of the close overlapping of the absorption curves for adenine, guanine, cytosine, and thymine, the precision obtained with such equations was poor for several selected wavelength sets tested. For this reason we resorted to a two-component analysis of the type described by LORING *et al.* The success of such an analysis depends upon a quantitative separation of the mixture into two pairs of substances, the purines and the pyrimidines. Solutions to the required simultaneous equations yielded the following general equations for any two-component mixture.

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General equations for determining the concentration of any pair of compounds, x and y

$$C_x = \frac{D_{w_2} - \frac{(e_{yw_2})\ (D_{w_1})}{e_{yw_1}}}{e_{xw_2} - \frac{(e_{xw_1})\ (e_{yw_2})}{e_{yw_1}}} \quad (1)$$

$$C_y = \frac{D_{w_2} - \frac{(e_{xw_2})\ (D_{w_1})}{e_{xw_1}}}{e_{yw_2} - \frac{(e_{xw_2})\ (e_{yw_1})}{e_{xw_1}}} \quad (2)$$

D is absorbancy (A_s)
w_1 is wavelength *one*
w_2 is wavelength *two*
x is compound *one*
y is compound *two*
e_x is molar absorbancy (a_M) of compound x
e_y is molar absorbancy (a_M) of compound y
C_x conc. of compound x in moles per liter
C_y conc. of compound y in moles per liter

Substitution of the molar absorbancy values given in Table III into eqns. (1) and (2) yielded the following eqns. from which the purine and pyrimidine concns. in moles per liter can be determined.

$$C_{(\text{cytosine})} = [1.38\,A_{s\,(280\,m\mu)} - 1.09\ \ A_{s\,(250\,m\mu)}] \cdot 10^{-4} \quad (3)$$

$$C_{(\text{thymine})} = [2.59\,A_{s\,(250\,m\mu)} - 0.804\,A_{s\,(280\,m\mu)}] \cdot 10^{-4} \quad (4)$$

$$C_{(\text{guanine})} = [2.45\,A_{s\,(280\,m\mu)} - 0.875\,A_{s\,(262\,m\mu)}] \cdot 10^{-4} \quad (5)$$

$$C_{(\text{adenine})} = [1.30\,A_{s\,(262m\mu)} - 1.48\ \ A_{s\,(280\,m\mu)}] \cdot 10^{-4} \quad (6)$$

IV. PROCEDURE FOR ANALYSIS OF DNA

A flow sheet outlining the analytical operations is given in Fig. 1.

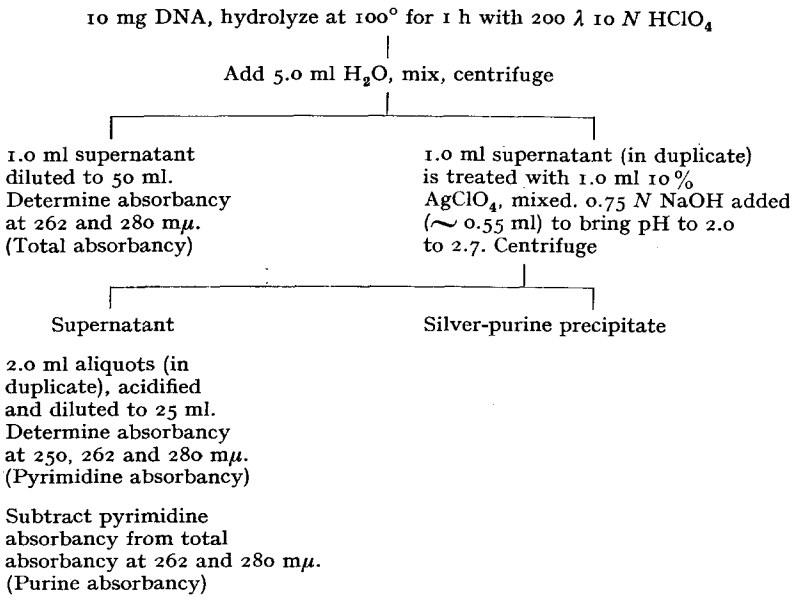

Fig. 1. Flow sheet of analytical operations.

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Nucleic acid hydrolysis

About 10 mg of DNA were weighed in a 15-ml conical centrifuge tube, and 200 λ of 10 *N* perchloric acid* were added. After a few minutes, the charred mass was well mixed by careful rotation, and kept at room temperature for 45 min until the nucleic acid was completely dissolved. The tube was stoppered, and heated for one hour at 100°. (The charred mass should not be disturbed during heating.) A blank containing only perchloric acid was similarly heated. After the tubes were cooled in a water bath, exactly 5.0 ml of water were added to each tube, and the charred mass was triturated with a stout glass rod.

Nucleic acid analysis

The stoppered tubes were centrifuged. 1.0 ml of the hydrolysate supernatant was removed with a syringe** and transferred to a 50-ml volumetric flask. After dilution to volume, the total absorbancy was determined, at 262 mμ and 280 mμ, against a properly prepared blank.

Two other 1.0 ml aliquots of the hydrolysate were transferred to 5-ml beakers. Another beaker was prepared with 1.0 ml of the heated blank solution. To each beaker was then added 1.0 ml (dispensed with another syringe) of a freshly prepared, 10% solution of silver perchlorate. Mixing was done with a small glass rod. A sufficient amount of 0.75 *N* NaOH ($\sim$ 0.55 ml) was then added (with stirring) to each beaker to bring its contents to pH 2.0 to 2.7 (checked on a pH meter with the outer surfaces of the electrodes carefully dried to avoid introducing volume errors). (Since the total volume of the mixture—in this case 2.55 ml—must be known, all volumes added must be measured with care.) The bulk (not quantitatively) of each solution was poured into a dry, conical centrifuge tube (15 ml). The tubes were stoppered and immediately centrifuged.

Exactly 2.0 ml of the supernatants, containing the pyrimidines alone, were transferred (by a 2 ml transfer pipette) to 25-ml flasks. After addition of one drop of concentrated perchloric acid, the flask contents were diluted to volume. The absorbancies of these solutions were determined against an appropriate blank at 250, 262, and 280 mμ. These data, representing the *pyrimidines alone*, were used in eqns. (3) and (4) to determine the pyrimidine content after appropriate dilution corrections.

Subtraction of the *pyrimidine values* (at 262 and 280 mμ) from the total absorption values (at 262 and 280 mμ) gave the absorption derived from purines which was used in eqns. (5) and (6).

Although light caused the silver purine precipitate to become mauve-colored, this was not deleterious, and was disregarded.

V. TEST OF METHOD WITH A PURINE AND PYRIMIDINE STANDARD

1. *Effect of pH on the quantitative separation of a known base mixture by means of silver perchlorate*

It was necessary to determine the optimum pH at which a silver perchlorate

* The exact strength of this acid was 9.737 *N*.

** 1 cc B-D Tuberculin syringe, smallest division, 0.01 cc. We have disregarded the difference between cubic centimeters and milliliters.

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solution precipitates purines quantitatively from solution without at the same time precipitating the pyrimidines.

Equimolar mixtures of adenine and guanine were precipitated with silver perchlorate reagent at various pH values. The absorbancy of the supernatants obtained by this treatment was measured against appropriate blank solutions at 262 mμ. Similarly, standard equimolar solutions of pyrimidines (thymine and cytosine) were subjected to the same treatment, and their optical absorbancies were determined at 280 mμ. Fig. 2 shows the result of such experiments in terms of the percentages of heterocyclic absorption still remaining in the supernatants after centrifugation of precipitates that formed. The data obtained in this manner are not precise since most of the absorbancy values fall at the extremes of the absorbancy range. However, it is clear from Fig. 2 that the optimum pH range for purine precipitation lies approximately between pH 2.0 and 2.7.

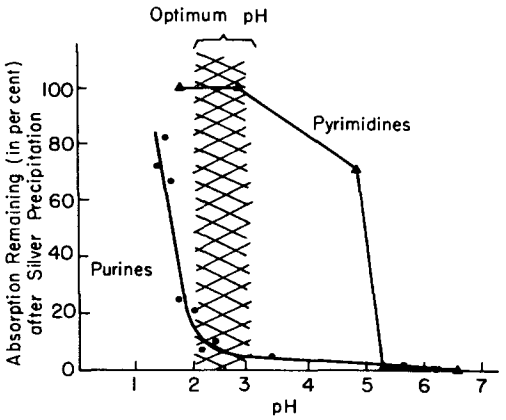

Fig. 2. Optimum pH for precipitation of purines with silver perchlorate.

2. Quantitative separation of a standard purine-pyrimidine mixture

In Table IV, typical data are given showing the quantitative separation of a known mixture of purines and pyrimidines at pH 2.1 to 2.2. Part A shows the *total absorption* of the four-component mixture at two wavelengths. These data were obtained in triplicate and are presented with their respective absorbancy ranges. Part B shows the *total pyrimidine absorption* of a similar standard mixture of pyrimidines (thymine and cytosine), at three wavelengths. Part C gives the absorbancy values found for the supernatant obtained after the same standard mixture of four bases that was used in Part A was treated with silver perchlorate at pH 2.1 to 2.2. A quantitative separation is obtained if the absorbancy values of Part C (supernatant) are identical with those values shown in Part B. Similarly, the absorbancy calculated for the purines (Part D) should equal the absorbancy found (Part E). The data in Table IV show that a quantitative separation of purines from pyrimidines is achieved under the conditions outlined in section IV within the limits of manipulative and spectrophotometric error demonstrated below.

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TABLE IV

EXPERIMENTAL AND STANDARD ABSORBANCY VALUES OBTAINED FROM THE QUANTITATIVE SEPARATION OF A STANDARD BASE MIXTURE

	Absorbancy values		
	250 mμ	*262 mμ*	*280 mμ*
Part A			
Total absorbancy of a guanine-adenine-thymine-cytosine standard	—	0.943 ± 0.003*	0.690 ± 0.007
Part B			
Absorbancy of a standard solution of pyrimidines (thymine + cytosine) of same concentrations as in Part A	0.241 ± 0.003	0.451 ± 0.003	0.419 ± 0.002
Part C (pyrimidines)			
Absorbancy of supernatant after addition of $AgClO_4$ to the solution used in Part A	0.248 ± 0.004	0.456 ± 0.003	0.421 ± 0.005
Part D			
Standard values for purines (*i.e.*, data of Part A minus data of Part B)	—	0.492	0.272
Part E (purines)			
Total absorption minus supernatant (pyrimidine) absorption (*i.e.*, data of Part A minus data of Part C)	—	0.487	0.269

* Maximum range of absorbancy values.

3. *Precision and accuracy of recovery of bases in a standard purine-pyrimidine mixture*

An approximately equimolar mixture of adenine, guanine, cytosine, and thymine ($2.4 \cdot 10^{-6}$ moles of each base per ml) was used to carry out a complete analysis as described in section IV. Particular care was taken to minimize random volumetric errors. The pH of purine precipitation was 2.0. Six separate determinations gave the absorbancy results shown in Table V. It is evident from these data that the maximum deviation in accuracy and precision is ± 0.8%.

TABLE V

PRECISION AND ACCURACY OF THE PURINE AND PYRIMIDINE SEPARATION PROCEDURE

	Pyrimidine absorbancy		
	250 mμ	*262 mμ*	*280 mμ*
Expected*	0.245	0.452	0.419
Found (average)**	0.244 ± 0.002	0.455 ± 0.001	0.420 ± 0.002
Error	— 0.4%	+ 0.7%	+ 0.2%
	Purine absorbancy		
	250 mμ	*262 mμ*	*280 mμ*
Expected*	—	0.497	0.275
Found (average)**	—	0.495 ± 0.002	0.274 ± 0.001
Error	—	— 0.4%	— 0.4%

* Expected on the basis of the molar absorbancy coefficients (Table III) and known concentrations.

** Six separate determinations.

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VI. TEST OF METHOD WITH NUCLEOTIDES AND NUCLEIC ACID

1. *Hydrolysis of nucleotides and nucleic acids*

To determine whether perchloric acid degrades purines and pyrimidines of nucleic acids, we exposed the crystalline deoxyribonucleotides to varying concentrations of perchloric acid for ½ h, at 100°, in stoppered tubes. Approximately $1.2 \cdot 10^{-5}$ moles of each nucleotide were hydrolyzed with 100 λ of perchloric acid of varying strengths (1 to 12 N). The absorbancies of the hydrolysates were then determined at two wavelengths. The results of these experiments are given in Fig. 3(a). The theoretical (calculated) absorbancies are indicated as dashed horizontal lines.

It would seem, from these experiments, that 100 λ of 5 or 6 N perchloric acid are optimum for the recovery of the expected absorbancy. When these hydrolysates were chromatographed on paper, it became evident that acid strengths of 3 N or stronger

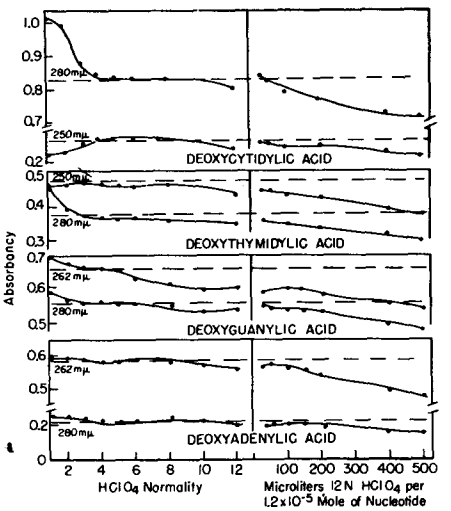

Fig. 3(a) and Fig. 3(b). The effect of perchloric acid on the absorbancy of deoxyribonucleotides.

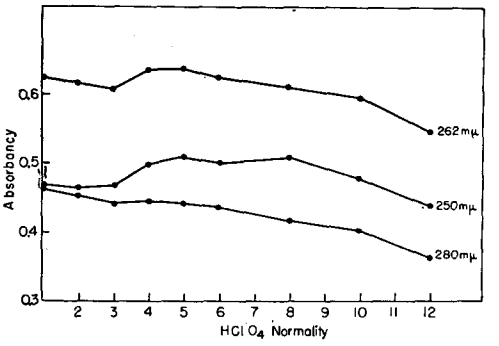

Fig. 4. The effect of various strengths of hot perchloric acid on the optical absorbancy of DNA.

caused extensive hydrolysis of the glycosidic bonds. 1 and 2 N perchloric acid solutions were not very effective in breaking the pyrimidine glycosidic bonds, and considerable amounts of thymidine and cytosine deoxyriboside were left in the hydrolysates.

Fig. 3(b) shows the results of experiments in which the strength of the perchloric acid was kept constant, but the volume of acid for each $1.2 \cdot 10^{-5}$ mole of nucleotide was increased from 30 λ to 500 λ. In these experiments it was found that excessive perchloric acid caused extensive destruction of both purines and pyrimidines, but particularly the latter. These results indicate that 100 λ of perchloric acid per 10^{-5} mole of nucleotide is about the maximum amount of 12 N $HClO_4$ which will give acceptable recoveries.

It is also evident that deoxyribose degradation products contribute no extraneous absorption to the hydrolysate in the region of higher acid strength.

In Fig. 4 the results of nucleic acid hydrolysis by different perchloric acid strengths are presented. In these experiments, 4.15 mg of DNA were hydrolyzed with 100 λ of

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perchloric acid solution, at 100°, for 30 min. In the complete hydrolysis of nucleic acids, *three* bonds, at least, must be hydrolyzed (for each nucleotide) instead of two bonds as in a free nucleotide. It seems likely that a larger amount of acid per nucleotide will be necessary for nucleic acid hydrolysis than for the free nucleotide.

Since the theoretical absorption of a nucleic acid hydrolysate cannot be accurately calculated, it is difficult to determine from Fig. 4 the optimum acid concentration for DNA hydrolysis. However, an examination of these hydrolysates by paper chromatography revealed that, besides the expected purines and pyrimidines, there were three new components, one with a bright blue fluorescence at R_F 0.48 (isopropyl alcohol–water–HCl); a second at R_F 0.34; and a third at R_F 0.69. The first, which is identical with the LEVY-SNELLBAKER[14] substance, gave an orange-tan color with diazotized sulfanilic acid; the second gave a purple-pink color; and the third, an orange color, with this reagent. These substances were especially evident in the hydrolysates produced by intermediate acid strengths (3 *N* to 8 *N*), and were absent from the very weak and strongly acid hydrolysates. Cytosine deoxyriboside and thymidine were also evident in the 1 *N* and 2 *N* perchloric acid hydrolysates.

It is not known whether these unknown components are artifacts arising from an intermediate stage of hydrolysis of DNA or are actually integral components of nucleic acids destroyed at the higher acid concentrations. They were present in considerable quantities in nucleic acids obtained from salmon sperm, from mouse liver, and from mouse hepatocarcinoma. Attempts are now being made to isolate them. It seems probable that the significantly higher absorption observed in the 4 *N* to 8 *N* acid hydrolysates is due to the absorption and stray light (fluorescence) contributed by the unknown materials.

Unfortunately, the above experiments do not clearly define the optimum conditions for hydrolysis of nucleic acids. The absorption curves indicate, however, that at least 10 *N* acid is necessary. An incomplete survey of the perchloric acid hydrolysis conditions recommended by other workers is given in Table VI.

TABLE VI

MICROLITERS OF PERCHLORIC ACID RECOMMENDED FOR EACH 10 mg DNA

Microliters of acid	*Normality*	*Literature*
143	12	15
52	12	16
200	7.5	17
80–150	12	18

2. DNA nitrogen–phosphorus recovery and base ratios obtained under varying conditions of hydrolysis

The criterion for optimum hydrolysis can be either nitrogen recovery, as calculated from the determined purine-pyrimidine content, or phosphorus recovery if one assumes that a strict one-to-one ratio exists between moles of base and moles of phosphorus.

DNA samples of 10.0 mg each (isolated from hepatocarcinoma tissue) were hydrolyzed for ½ h, at 100°, with several different strengths of perchloric acid (200 λ). Purine precipitation with silver perchlorate was carried out at pH 2.0. The results

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of these experiments are given in Table VII. These data show that hydrolysates prepared with dilute acid contain, in addition to the four desired purines and pyrimidines, other products, presumably of an intermediate stage of hydrolysis. Thus, the calculated nitrogen and phosphorus values (upper parts of columns 9 and 10, Table VII) based on eqns. (3), (4), (5), and (6) deviate very widely from the direct nitrogen and phosphorus determinations. It is evident that the use of either 10 or 12 *N* perchloric acid yields a quantitative base recovery and nearly theoretical base ratios (lower parts of columns 6 to 10, Table VII). However, further investigation showed that 12 *N* perchloric acid sometimes resulted in aberrant base ratios, and could not always be relied upon to yield complete recoveries. The reasons for this are not well defined, but it is certain that base destruction can occur with some nucleic acid samples under what appears to be inadequately controlled hydrolysis conditions. Because of this difficulty, an attempt was made to define the optimum conditions for hydrolysis with 10 *N* perchloric acid. From the data shown in Table VIII, it was

TABLE VII

EFFECT OF $HClO_4$ STRENGTH ON HETEROCYCLIC BASE RECOVERY AND RATIOS

Perchloric acid normality	*Cytosine*	*Thymine*	*Adenine*	*Guanine*	*Cytosine / Guanine*	*Adenine / thymine*	*Purines / Pyrimidines*	*Moles of phosphorus acc'd for**	*Nitrogen acc'd for (mg)**
	**	**	**	**					
4	13.0	29.2	21.3	28.6	0.356	1.37	1.38	$2.90 \cdot 10^{-6}$	0.152
5	12.9	32.1	19.3	35.8	0.362	1.66	1.22	$2.82 \cdot 10^{-6}$	0.197
6.5	14.4	35.4	20.6	29.4	0.489	1.72	1.01	$2.83 \cdot 10^{-6}$	0.145
7	15.3	37.6	22.4	24.2	0.622	1.68	0.89	$2.67 \cdot 10^{-6}$	0.134
8	16.8	36.5	25.3	21.3	0.791	1.44	0.87	$2.46 \cdot 10^{-6}$	0.122
10	19.6	29.6	30.3	20.5	0.96	0.98	1.03	$2.46 \cdot 10^{-6}$	0.128
12	21.2	29.6	28.8	20.4	1.04	1.02	1.03	$2.46 \cdot 10^{-6}$	0.127
				Direct determination of phosphorus				$2.47 \cdot 10^{-6}$	
				Direct determination of nitrogen					0.1264

* Phosphorus and nitrogen "accounted for" were calculated on the basis of the mole contents given in columns 2, 3, 4, and 5.

** Moles of base $\times 10^{-7}$/mg NaDNA.

TABLE VIII

EFFECT OF DIGESTION TIME ON HETEROCYCLIC BASE RECOVERY (IN MOLES OF HETEROCYCLIC BASE PER mg NaDNA)

	Digestion times at 100°			
	½ h	*1 h*	*2 h*	*3 h*
Cytosine	$5.85 \cdot 10^{-8}$	$5.81 \cdot 10^{-8}$	$5.73 \cdot 10^{-8}$	$5.80 \cdot 10^{-8}$
Thymine	$7.23 \cdot 10^{-8}$	$7.08 \cdot 10^{-8}$	$6.80 \cdot 10^{-8}$	$6.50 \cdot 10^{-8}$
Adenine	$7.76 \cdot 10^{-8}$	$7.26 \cdot 10^{-8}$	$7.38 \cdot 10^{-8}$	$7.22 \cdot 10^{-8}$
Guanine	$5.73 \cdot 10^{-8}$	$5.85 \cdot 10^{-8}$	$5.31 \cdot 10^{-8}$	$5.76 \cdot 10^{-8}$
Total	$26.6 \cdot 10^{-8}$	$26.0 \cdot 10^{-8}$	$25.2 \cdot 10^{-8}$	$25.3 \cdot 10^{-8}$
Calculated	$25.9 \cdot 10^{-8}$	$25.9 \cdot 10^{-8}$	$25.9 \cdot 10^{-8}$	$25.9 \cdot 10^{-8}$
Adenine / Thymine	1.07	0.99	1.07	1.11
Guanine / Cytosine	0.98	1.00	0.93	1.00
Purines / Pyrimidines	1.03	1.02	1.01	1.06

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TABLE IX

MOLE-% COMPOSITION OF SALMON NUCLEIC ACIDS

Sperm sample	Mole-per cent of recovered base				Adenine / Thymine	Guanine / Cytosine	Purines / Pyrimidines	Moles phosphor per 10 mg DN
	Cytosine	Thymine	Adenine	Guanine				
Silver salmon	21.6	27.6	28.8	21.8	1.04	1.01	1.03	$2.48 \cdot 10^{-5}$
Chinook salmon	22.4	27.2	27.9	22.5	0.99	1.00	1.02	$2.59 \cdot 10^{-5}$
Chum salmon	21.9	27.6	28.7	21.9	1.01	1.00	1.02	$2.37 \cdot 10^{-5}$
Sockeye salmon	21.8	27.8	28.2	22.0	1.01	1.01	1.01	$2.59 \cdot 10^{-5}$
Humpback salmon	22.6	27.5	27.7	22.5	1.01	1.01	1.01	$2.52 \cdot 10^{-5}$

decided that optimum conditions obtained when 10 mg of DNA were hydrolyzed with 200 λ of 10 N perchloric acid, at 100°, for 1 h. Longer periods of digestion caused noticeable destruction of thymine.

VII. APPLICATION OF PROCEDURE TO ANALYSIS OF SALMON SPERM NUCLEIC ACID

The conditions given in section IV were applied to the analysis of sperm nucleic acids obtained from five species of Pacific Ocean salmon (Table IX). These data show that unitary base ratios and nearly complete base recovery (98–100%) are achieved.

VIII. DISCUSSION

The reliability of a procedure for the analysis of purines and pyrimidines of nucleic acids is generally based on nitrogen or phosphorus recoveries. Unfortunately, a check based on these constituents is not completely satisfactory. An accounting of all the nitrogen in terms of the four constituent bases is not possible, usually because almost all nucleic acid samples contain small amounts of foreign nitrogen, mainly protein, which is very difficult to remove. For this reason we have not, as a rule, relied upon total nitrogen analyses. Recoveries based on total phosphorus are likewise unsuitable because two assumptions must be made: (1) that no foreign phosphorus-containing substances (*e.g.*, RNA or phospholipids) are present; and (2) that nucleic acids contain, according to theory, a strict one-to-one ratio between moles of heterocyclic base and phosphorus. Few, if any, nucleic acid preparations of mammalian origin have been entirely free of ribonucleic acid. In the second assumption, it is implied that no triply esterified or secondary phosphoryl groups are present.

It is well established that fish sperm nuclei contain very small amounts, if any[19], of ribonucleic acid, and the protamine associated with the nucleic acid is readily eliminated by standard procedures. Hence, fish sperm nucleic acids are well suited for testing the quantitativeness of base recoveries. Table IX gives a complete purine and pyrimidine recovery, based on phosphorus analyses, for sperm nucleic acids of five species of salmon. The recoveries shown in the last column ranged from 98 to 100%.

The strongest argument in favor of the WATSON-CRICK model for DNA structure depends upon the adenine–thymine and guanine–cytosine ratios. According to this model, the integrity of the double-stranded DNA molecule depends upon hydrogen bonding between the base pairs that occupy adjacent positions in opposing nucleotide chains. Although such pairing requires that the ratios be unitary, reports of large

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Total moles base found	*Per cent recovered (based on phosphorus)*
$2.47 \cdot 10^{-5}$	100
$2.60 \cdot 10^{-5}$	100
$2.34 \cdot 10^{-5}$	99
$2.54 \cdot 10^{-5}$	98
$2.51 \cdot 10^{-5}$	100

deviations are still appearing. Because of our initial failure to obtain values of unity for the ratios, we were led to reinvestigate the hydrolysis procedure. It now seems certain that non-unitary ratios obtained in our earliest experiments resulted from base destruction (particularly of thymine) during hydrolysis. In this connection it is important to note that the analyses which gave unitary ratios also gave the most quantitative recovery in terms of total nitrogen or phosphorus. We therefore believe that ratios deviating more than a few % from unity are the result of selective destruction of bases during hydrolysis. On the whole, our analyses on sperm nucleic acids do not conflict with the WATSON-CRICK theory.

The quantitative recovery obtained by our procedure suggests that the three unknown substances that we found in hydrolysates prepared with perchloric acid of intermediate strengths are actually artifacts arising during nucleic acid degradation. If these substances are real DNA components, they must be either triply esterified to phosphorus or united to the nucleic acid in some other way which does not involve phosphorus at all. Although the latter alternatives seems unlikely, we are now attempting to find these unidentified substances in enzymic hydrolysates of nucleic acids.

SUMMARY

1. A spectrophotometric method for the analysis of nucleic acid purines and pyrimidines is described. Data which illustrate the optimum conditions for perchloric acid hydrolysis of DNA are presented. Heterocyclic base recovery in terms of phosphorus or nitrogen is 98–100 % complete.

2. Purine and pyrimidine contents of five species of salmon sperm DNA were studied. Hydrolysis conditions which gave complete recovery in terms of phosphorus also yielded unitary values for the following base ratios: adenine/thymine, guanine/cytosine, and purine/pyrimidine.

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