PURINE AND PYRIMIDINE CONTENTS OF SOME DESOXYPENTOSE NUCLEIC ACIDS

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In this paper the pyrimidine and purine contents of nucleic acids of the desoxypentose type are given. The nucleic acids analyzed were prepared from different tissues of the same organism and from a variety of different organisms. Preparations from eleven different sources were studied. In many of the preparations it was possible to account for all of the nucleic acid phosphorus present; and in every case at least 92.5 per cent was accounted for.

We have previously described a chromatographic method for the quantitative separation of purines and pyrimidines on a starch column (1). To determine these bases in nucleic acids hydrolytic procedures are required which release all the bases present and yet do not at the same time destroy any of them. Such procedures are described in this paper.

The analytical results obtained have a bearing on the following problems: on whether any bases other than adenine, guanine, thymine, and cytosine are present in desoxypentose nucleic acids; on the tetranucleotide hypothesis; and on whether nucleic acids from different sources differ in their purine-pyrimidine composition. To clarify these problems it is, of course, important that analysis of the nitrogenous constituents of a nucleic acid should account for all the phosphorus present. This has not previously been accomplished. In the most recent work on these problems yields of from 76 to 90 per cent have been obtained (2, 3).

Separation of the Bases.—The separation of the bases was accomplished by chromatography on starch columns (1). The effluent was collected in a series of small fractions, and the amount of base in each fraction was determined by ultraviolet spectroscopy. On known mixtures recoveries have averaged 100 ± 4 per cent. A typical separation of a known mixture containing seven components is shown graphically in Fig. 1.

Because of the possibility that hydrolysates containing nucleosides might be encountered, experiments were carried out to determine whether nucleosides would be separated from the free bases in the procedure employed. The desoxypentose nucleosides of thymine and guanine were the only ones readily available for studies of chromatographic behavior. Consequently, the pentose

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nucleosides of adenine and cytosine were used assuming that their behavior would approximate that of desoxypentose nucleosides. It was found that pyrimidine nucleosides were not completely separated from the free bases (Fig. 2). The presence of a mixture of pyrimidines and their nucleosides in the hydrolysates, however, would result in at least a broadening of the peaks in the thymine and cytosine positions. The separation of the purines from their nucleosides was even less satisfactory. Adenosine and guanosine moved

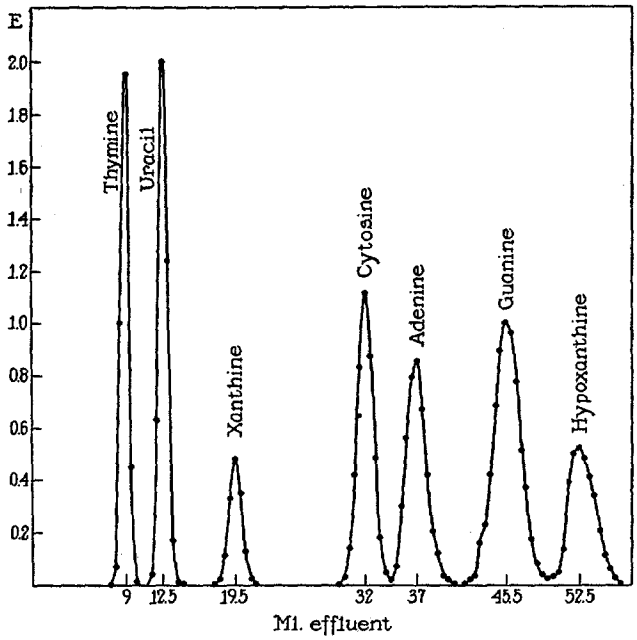

FIG. 1. Chromatographic separation of a synthetic mixture containing seven bases. Solvent 2:1 *n*-propanol—0.5 N HCl. Column, 10.9 gm. of starch (anhydrous); diameter, about 0.8 cm.; height, about 30 cm.

at approximately the same rate as guanine, the three substances forming a single broad peak in the guanine position. However, examination of the absorption spectrum of the material in the guanine peak provides an indication of its homogeneity.

Preparation of Nucleic Acids.—Most of the nucleic acids used in our work were prepared by a procedure which depends upon the knowledge that desoxypentose nucleoproteins are insoluble in physiological saline but soluble in concentrated neutral saline (4). In this way desoxypentose nucleic acids can be prepared from many sources and the danger of autolytic changes is slight. The other method for preparing polymerized desoxypentose nucleic acid cur-

rently in use is applicable to the thymus gland only and quite possibly involves autolytic changes, for a prolonged aqueous extraction is required (5). By the procedure we have employed the bulk of the desoxypentose nucleic acid of the tissue is extracted in all cases with the possible exception of the wheat germ.

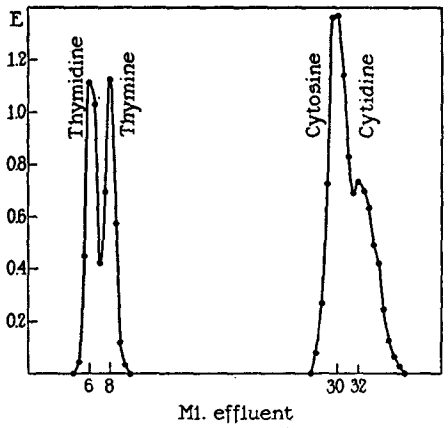

FIG. 2. Chromatographic separation of a synthetic mixture containing thymine, thymidine, cytosine, and cytidine. Solvent 2:1 *n*-propanol—0.5 N HCl. Column, 10.9 gm. of starch (anhydrous); diameter, about 0.8 cm.; height, about 30 cm.

The nucleic acids of calf kidney, sheep spleen, horse spleen, and shad testes are prepared by identical procedures, all in a cold room at 2°C. The fresh tissues are minced with scissors and then placed in the Waring mixer with a salt mixture, 0.14 M sodium chloride and 0.01 M sodium citrate, the latter added to inhibit the action of desoxyribonuclease. In a typical experiment the mixer contains 150 gm. of tissue, 350 cc. of saline-citrate, and 150 gm. of crushed ice. The mixer is run for 6 minutes, and at the end its contents are still ice cold. They are strained through a double layer of gauze and then through a fine towel. The strained fluid is centrifuged and the turbid supernatant discarded. The sediment is washed several times with saline-citrate, so that most of the ribose nucleoproteins present are removed.

In the next step the sediment is suspended in 1 M NaCl (final concentration) and the desoxypentosenucleic acid is dissolved. The first sign that this is happening is a striking increase in viscosity. The viscous mixture is stirred at high speed either in the Waring mixer or by a well centered stirrer running at 8,000 to 15,000 R.P.M. Vigorous stirring is needed to break the lumpy gel. If the stirring is sufficiently vigorous, the process is completed in about 5 minutes. The volume to which this mixture is brought depends upon its viscosity; it must be fluid enough to be clarified by centrifugation at 12,000 to 17,000 R.P.M.

Nucleoprotein is precipitated from the supernatant by adding it to 6 volumes of water. The fibrous precipitate is collected around a rod with a crook at one end and any non-adherent precipitate is discarded. The fibrous material is dissolved in 1 M

NaCl. The viscous fluid is centrifuged at high speed and the sediment is discarded. Nucleoprotein is again precipitated by adding to 6 volumes of water. This process is repeated twice more.

Protein is removed by shaking the viscous 1 M NaCl solution with an octyl alcohol–chloroform mixture (1 volume octyl alcohol to 4 volumes chloroform). Removal of protein occurs more rapidly and more completely if the nucleoprotein solution is made alkaline. Enough sodium hydroxide is added to the 1 M NaCl solution so that when 0.25 cc. of it is added to 1.5 cc. of water a fibrous precipitate just fails to form, pH between 9.2 and 9.8. "Shaking" is most rapidly accomplished in the Waring mixer. To 2 volumes of the 1 M NaCl is added 1 volume of octyl alcohol–chloroform. This is vigorously mixed for 12 minutes and then centrifuged. The clear supernatant is again mixed with octyl alcohol–chloroform. The process is repeated until no more protein appears at the water-chloroform interface.

Nucleic acid is precipitated from 1 M NaCl solution by adding this to 3 volumes of alcohol. The fibrous precipitate of nucleic acid is wound round a rod and the bulk of the fluid pressed from it. With forceps nucleic acid is picked off the rod as a mass of small fibers. They are washed briefly in 64 per cent alcohol to remove sodium chloride, then dehydrated with alcohol and dried at room temperature after removing the alcohol with ether.

In preparing desoxyribosenucleic acid from the thymus the procedure that has just been described can be followed. We find it preferable, however, first to isolate thymus chromosomes (6) and then prepare nucleic acid. The isolated chromosomes, suspended in 1 M NaCl are stirred at high speed and the clear supernatant after centrifugation at 17,000 R.P.M. is made alkaline and deproteinized by mixing with octyl alcohol–chloroform.

It is also possible to extract nucleic acid from isolated chromosomes with sodium desoxycholate. The chromosomes are suspended in 0.5 per cent (final concentration) sodium desoxycholate, rapidly stirred, and then centrifuged. When the supernatant is added to 3 volumes of alcohol a fibrous precipitate forms. This is pressed free of alcohol and dissolved in water. Enough sodium chloride is added to bring the concentration to 1 M and the solution is then deproteinized.

The nucleic acid of turtle erythrocytes is prepared from isolated chromosomes. Washed erythrocytes, suspended in saline-citrate, are passed through a colloid mill. The fragmented cell material is centrifuged at low speed. The sediment consisting of chromosomes, is washed free of hemoglobin with saline-citrate and then suspended in 1 M NaCl and stirred rapidly. This material is centrifuged at high speed. The supernatant is made alkaline and deproteinized. The nucleic acid of fowl erythrocytes is prepared in the same way, the only difference being that after passing the erythrocytes through the colloid mill, nuclei, many of them drawn out, are obtained instead of chromosomes.

Desoxypentose nucleic acid is prepared from wheat germ in much the same way as it is from kidney or spleen. One difference is that the wheat germ (which should be fresh and unheated) must first be extracted thoroughly with petrol ether. Another difference is that the extract in 1 M NaCl should be added to 10 volumes of water, instead of to 6 volumes. In other respects, the procedure for wheat germ is the same as that described for the kidney.

In preparing desoxypentose nucleic acid from sperm of the sea urchin, *Arbacia punctulata*, several differences are to be noted. The sperm, washed in sea water, are extracted with 2 M NaCl, instead of, as in other cases, with 1 M NaCl. A fibrous precipitate is obtained on adding this extract to 3 volumes of water.

Preparation of desoxypentose nucleic acid from trout sperm is simpler than from any other source (7). The sperm are suspended in 1 M NaCl, stirred rapidly, and then centrifuged at high speed. The clear supernatant is dialyzed against 1 M NaCl for several weeks, long enough to remove all protamine. On adding this solution to 3 volumes of alcohol, nucleic acid precipitates.

All the nucleic acid preparations that have been described gave negative tests with the Millon reagent, indicating the absence of gross protein contamination. The preparations were also free of pentose nucleic acid as no uracil was found in the hydrolysates.

A preparation of desoxypentose nucleic acid from Type III pneumococcus, containing the "transforming principle," was kindly given to us for analysis by Dr. R. D. Hotchkiss.

Hydrolytic Procedures.—The action of concentrated acid at relatively high temperatures is required for the liberation of pyrimidines from desoxypentose nucleic acids; purines, on the other hand, are released by mild hydrolytic procedures and are destroyed by strong acid.

Liberation of Pyrimidines.—Hotchkiss (8) studied the hydrolysis of yeast nucleic acid at 120° for 2 hours with various concentrations of HCl. At the lower concentrations of acid pyrimidine nucleosides were found in the hydrolysates, their amounts decreasing with increasing acid concentration until the free pyrimidines finally appeared.

In this work 10 mg. portions of nucleic acid were hydrolyzed with 2 ml. of 6 N HCl for 2 hours at 120°C. The hydrolysate was evaporated to dryness *in vacuo* and dissolved in 4 to 5 ml. of 2:1 *n*-propanol–0.5 N HCl. One ml. portions of the final solutions were placed on the columns for chromatographic analysis. The result of a fractionation of a hydrolysate of thymus desoxyribonucleic acid in 6 N HCl is shown graphically in Fig. 3.

There is good evidence that the liberation of pyrimidines is quite complete under these conditions. The sharpness of the thymine and cytosine peaks on the chromatogram indicates the absence of appreciable amounts of nucleosides. Furthermore, the ultraviolet absorption spectra in acid and alkali of the pyrimidines recovered from the hydrolysates correspond very closely to those of known solutions of the compounds, and are quite distinct from those of the corresponding nucleosides. Increasing the temperature of the hydrolysis 10 or 20° did not increase the yield of pyrimidines, as was shown in the case of a wheat germ preparation.

There is no evidence of destruction of the pyrimidines under these conditions of hydrolysis. Thymine and cytosine were the only pyrimidines found in the hydrolysates. It has been reported that cytosine is converted to uracil

upon treatment with strong mineral acids at high temperatures (9). Under the conditions used here no such conversion was observed, and control experiments with known mixtures yielded quantitative recoveries of both thymine and cytosine. Cytosine could also be recovered quantitatively when treated under the hydrolytic conditions in the presence of nucleic acid (from wheat germ). The only artifact found consistently in all hydrolysates in 6 N HCl was xanthine formed by the deamination of guanine. Xanthine was identified by its absorption spectrum and position on the chromatogram.

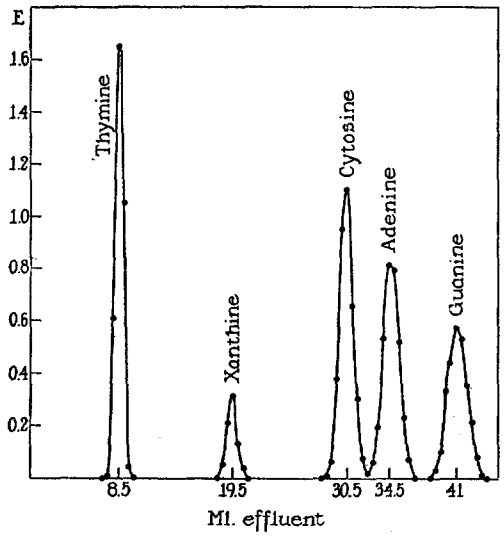

FIG. 3. Chromatographic separation of a hydrolysate of thymus desoxyribonucleic acid in 6 N HCl. Solvent 2:1 *n*-propanol—0.5 N HCl. Column, 10.9 gm. of starch (anhydrous); diameter, about 0.8 cm.; height, about 30 cm.

Liberation of Purines.—In our initial experiments a procedure similar to that used by Hotchkiss (8) was employed. This involved heating the nucleic acids in 0.5 N HCl at 120° for 2 hours. Under these conditions quantitative recoveries could not be obtained consistently with known mixtures of adenine and guanine, and the purines were liberated in relatively low yield from the nucleic acids. Consequently other procedures were sought. The method of Levene (10) consisting of the passage of HCl gas into a suspension of nucleic acid in 95 per cent methanol did not prove to be suitable. Li and Stacey (11) reported that purines are released from desoxypentose nucleic acids on standing in 1.5 per cent methanolic HCl at room temperature. This procedure appeared to offer a possibility of complete liberation of purines with a minimum of destruction.

This method was applied and was found to produce the highest yields of purines which we had obtained up to that time, while control experiments with known mixtures yielded quantitative recoveries of adenine and guanine. Approximately 2 days was required to produce the maximum yield of purines. In order to shorten the time of hydrolysis the effects of increased temperature and acid concentration were investigated. Twenty hours was chosen as a convenient time interval, and at 37°, using 1.5 per cent HCl, this was sufficient to bring about maximum hydrolysis. Additional evidence that the hydrolysis is complete under these conditions is given by the fact that the use of 3 per cent HCl failed to increase the yield, while lower concentrations of HCl produced lower yields. In methanolic HCl adenine is released more rapidly than guanine,

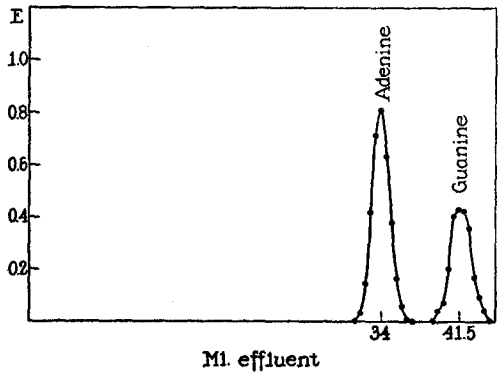

FIG. 4. Chromatographic separation of the supernatant of a hydrolysate of thymus desoxyribonucleic acid in 1.5 per cent methanolic HCl. Solvent 2:1 *n*-propanol—0.5 N HCl. Column, 10.9 gm. of starch (anhydrous); diameter, about 0.8 cm.; height, about 30 cm.

and the liberation of both is complete before appreciable amounts of phosphorus are liberated.

The procedure finally employed was to treat 10 mg. portions of nucleic acid with 5 ml. of 1.5 per cent methanolic HCl (prepared by diluting concentrated aqueous HCl with methanol) for 20 hours at 37°C. A sample of the supernatant was removed and evaporated to dryness *in vacuo*. The residue was dissolved in 2:1 *n*-propanol–0.5 N HCl and subjected to chromatographic analysis. No pyrimidines were released by this procedure, and adenine and guanine were the only bases demonstrated on the chromatograms (Fig. 4).

The presence of nucleosides in the hydrolysates would introduce an error in the determination of guanine, but it is unlikely they were present. The absorption spectra in acid and alkali of the material in the guanine peaks corresponded very closely to those of known solutions of guanine and were

quite distinct from those of the nucleoside. In the hydrolysis of desoxypentose nucleic acid under these conditions there is not much chance of the formation of nucleosides, as the bond between purine and sugar is so easily broken by acid. Enzymatic hydrolysis is required for the preparation of purine nucleosides from these nucleic acids.

Analytical Results.—The nucleic acids which have been analyzed came from a wide variety of sources. First preparations from different tissues (thymus and kidney) of the same animal, the calf, were studied. Then we investigated

TABLE I

The Distribution of Purines and Pyrimidines in Desoxypentose Nucleic Acids

Nucleic acid	N	P	N/P	Moles per 100 moles P				P accounted for
				Adenine	Guanine	Thymine	Cytosine	
	per cent	*per cent*						*per cent*
Calf thymus I	14.7	8.19	1.80	27.6	23.5	28.0	19.7	98.8
Calf thymus II	14.2	8.24	1.72	26.6	21.7	28.6	20.1	97.0
Calf thymus (desoxycholate)	14.7	8.07	1.82	28.1	23.5	28.2	20.4	100.2
Calf kidney	14.1	8.13	1.73	27.4	21.9	27.3	20.3	96.9
Horse spleen	15.9	8.36	1.90	28.3	21.9	26.3	19.2	95.7
Sheep spleen	14.2	8.71	1.63	26.0	20.7	26.6	19.6	92.9
Chicken erythrocyte	15.6	8.76	1.78	27.0	19.2	27.4	20.1	93.7
Turtle erythrocyte	13.9	7.91	1.76	29.9	22.9	29.1	22.2	104
Trout sperm	14.6	7.69	1.90	29.4	22.2	27.1	19.9	98.6
Shad testes	14.4	8.20	1.76	26.4	20.3	27.2	19.0	92.9
Sea urchin sperm	12.6	7.54	1.67	26.7	18.3	30.8	18.1	93.9
Wheat germ	14.3	8.08	1.77	27.4	23.6	27.0	16.4	94.4
Pneumococcus Type III			1.72	27.6	19.0	29.2	16.7	92.5

The phosphorus and nitrogen contents were determined on the original nucleic acid preparations except in the case of the pneumococcus preparation for which the phosphorus was determined on the hydrolysate. The nitrogen-phosphorus ratio for this preparation is the value reported by Hotchkiss (16).

nucleic acids prepared from a closely related mammal—the sheep, a less closely related mammal—the horse, and finally from a number of more distantly related organisms—a bird (the domestic fowl), a reptile (the turtle), fishes (the trout and shad), an echinoderm (the sea urchin), a higher plant (wheat), and a microorganism (pneumococcus).

The results of the analyses are summarized in Table I. The percentage compositions are average values calculated from the chromatographic analyses of at least two hydrolysates for each preparation with the exception of the pneumococcus where sufficient amounts of material were not available. The composition of the nucleic acids with respect to purines and pyrimidines is expressed as the mole per cent of nucleic acid phosphorus, and the total

recoveries are also expressed on the basis of phosphorus. This method of expressing recoveries has been used by other investigators (2, 3, 9). The calculation of the yield on this basis involves the assumption that the nucleic acid is a polynucleotide with one mole of phosphorus per mole of base. The polynucleotide structure has been well established in the case of thymus desoxypentose nucleic acid (12). If the calculation of the yields is made on the basis of nitrogen, no assumption is made as to the structure. The question arises as to whether the nitrogen or phosphorus content of a preparation is the more reliable basis for a calculation of total recoveries.

The analytical procedures for the determination of nitrogen and phosphorus in the preparations do not distinguish between true nucleic acid nitrogen and phosphorus and that which is present as an impurity. The principal phosphorus compounds which might conceivably be present as contaminants are inorganic phosphate, nucleotide, sugar phosphate, lipid, and pentose nucleic acid. The procedures used in the preparation of the nucleic acids should eliminate these substances. The nucleic acids are precipitated from aqueous solutions by 64 per cent alcohol; the inorganic phosphorus, sugar phosphates, and nucleotides are soluble under these conditions. Lipids are removed by extractions with alcohol and ether. It has already been mentioned that the absence of uracil in the hydrolysates indicates that the preparations were free of pentose nucleic acid. On the other hand, it is quite possible that the nucleic acids were contaminated with protein. Protein is chemically bound to nucleic acid in tissues and is removed with difficulty. Even after repeated separations some protein may remain in combination with the nucleic acid, and there is at present no way of being certain of its complete removal or of how much remains combined.

For these reasons phosphorus-containing impurities are less likely to be present than those containing nitrogen, and the analytical values for phosphorus are considered to be a better criterion for the nucleic acid content of a preparation than the nitrogen values.

The recoveries of nucleic acid phosphorus are nearly quantitative in about half of the preparations; in others it is never less than 92.5 per cent. The lower recoveries may be the result of the presence of unidentified components, or of the incomplete liberation or partial destruction of the bases in the course of the hydrolysis. Our experience has shown that low yields will be obtained if a preparation is a poor one from certain points of view such as the presence of protein.

The analyses indicate that adenine, guanine, thymine, and cytosine are certainly the major nitrogenous constituents of desoxypentose nucleic acids as has been supposed for a long time (12). The possibility of the presence of considerable amounts of other substances was not eliminated even in the most recent work on this problem, where from 76 to 90 per cent of nucleic acid

phosphorus was accounted for (2, 3). Other materials might have been destroyed in the course of the hydrolysis or escaped detection because their nature was unknown. Our work has made it possible to conclude that no more than traces of other constituents could be present in those nucleic acids for which a quantitative recovery was made.

Until recently a generally accepted view of the composition of desoxypentose nucleic acids was expressed in the tetranucleotide hypothesis according to which the four bases are distributed in equimolecular proportions in the molecule. This hypothesis is based principally on the work of Steudel (13) and of Levene and Mandel (14). Gulland (15) and Chargaff (2) and their coworkers have recently challenged this hypothesis. The latter group of investigators made their conclusions on the basis of molecular ratios calculated from their data on the percentage composition of the desoxypentose nucleic acids of thymus and spleen. These molecular ratios may be in error as they were calculated on the basis of incomplete recoveries of nucleic acid phosphorus (about 90 per cent) assuming that hydrolysis losses were distributed equally among the four bases. We have observed that in the hydrolysis of desoxypentose nucleic acids with 0.5 N HCl at 120°, where some destruction of purines takes place, relatively more adenine than guanine is destroyed. Therefore, it is not safe to assume that hydrolysis losses will affect equally all the components. The yield of adenine reported by Chargaff *et al.* was, however, somewhat higher than the value required for a tetranucleotide. The results which are presented here provide further evidence that the tetranucleotide hypothesis is no longer tenable. The evidence is stronger in the case of those preparations in which the recoveries of phosphorus were quantitative, but in no case does the composition permit an equimolecular distribution.

The assumption that all desoxyribonucleic acids have the same composition is implicit in the tetranucleotide hypothesis. Up to this time preparations from relatively few sources have been analyzed. Vischer *et al.* (3) have recently presented data for the percentage composition of the desoxypentose nucleic acids of yeast and the tubercle bacillus, which together with other data for the nucleic acids of calf thymus and beef spleen (2), tend to indicate differences in composition. The total recoveries of nitrogen for the yeast and tubercle bacillus preparations were, however, somewhat low—about 76 per cent. Our values for the percentage composition of thymus nucleic acid are similar to those obtained by Chargaff *et al.* (2) for the same nucleic acid, but the pyrimidine yields are somewhat higher increasing the total recovery. The results which we obtained for the three samples of this nucleic acid, one of which was prepared by a different procedure, are in good agreement.

The general distribution of purines and pyrimidines in all the preparations examined is rather similar. In most cases the differences observed are too small to be considered significant. In the case of those preparations where the dis-

tribution appears to vary, the apparent difference in composition is confined to only one of the four bases and the total recoveries are somewhat low. For the wheat germ and pneumococcus preparations the yield of cytosine is lower than for the other nucleic acids, and the total recoveries are 94.4 and 92.5 per cent respectively. The amounts of the other bases, however, are very close to those found in a majority of the preparations. These considerations lead one to suspect that even these apparently large differences in the quantity of cytosine may not be significant.

The low yields of cytosine may be the result of the destruction of this base in the course of hydrolysis or of incomplete hydrolysis. It is probably not the result of incomplete hydrolysis in the case of the wheat germ, as increasing the temperature of the hydrolysis 10 or 20° did not increase the yield of cytosine. It was not possible to make further studies of the hydrolysis of the pneumococcus preparation as sufficient amounts of material were not available. An indication that there may have been some destruction in the course of the hydrolysis of this preparation is provided by the fact that the hydrolysate contained a black, humin-like precipitate. Such extensive humin-like precipitation was not observed in the hydrolysates of highly purified nucleic acid preparations.

It should also be mentioned that an additional peak was observed on the chromatogram of the hydrolysate of the wheat germ in 6 N HCl. This peak was not observed in any other hydrolysate and has not yet been identified. It might represent another component or a decomposition product of cytosine. Although such a product was not observed when cytosine was treated under the same hydrolytic conditions, it might be formed under the influence of impurities in the wheat germ preparation.

The relative amounts of adenine, thymine, and cytosine in the desoxypentose nucleic acid from Type III pneumococcus, containing the "transforming principle," were reported by Hotchkiss (16). Our value for adenine is much higher than his. When we employed hydrolytic procedures similar to those used by Hotchkiss, we found that as much as 20 per cent of the adenine in thymus nucleic acid is destroyed. From his ratio of thymine to adenine the yield of adenine in his experiments can be calculated assuming that his yield of thymine was the same as ours. This is a reasonable assumption, as the hydrolysis procedures which we employed for the liberation of pyrimidines were very similar to his. If this is done, it appears that about 30 per cent of the adenine was lost in his experiments. These considerations indicate that there is no sure foundation for his assertion that the results of his analyses show differences in composition of nucleic acids from different sources.

A comparison of the chemical constitution of these macromolecular compounds must be based on information as to the nature and proportions of their components, their arrangement in the molecule, and the types of linkages holding them together. This investigation has been limited to a com-

parison of the distribution of purines and pyrimidines in the compounds. The results of the analyses of a wide variety of nucleic acids have shown no great differences in this respect.

SUMMARY

The distribution of purines and pyrimidines in desoxypentose nucleic acids prepared from a variety of animal and plant sources has been studied.

1. The nucleic acids were prepared from calf thymus, calf kidney, sheep spleen, horse spleen, chicken erythrocyte, turtle erythrocyte, trout sperm, shad testes, sea urchin sperm, wheat germ, and Pneumococcus Type III.

2. Separate hydrolyses were carried out for the determination of purines and pyrimidines. These procedures permitted nearly quantitative recovery of nucleic acid phosphorus in many of the preparations examined.

3. In the case of those preparations where a quantitative recovery was obtained it can be concluded that no bases other than adenine, guanine, thymine, and cytosine were present in appreciable amounts.

4. The distribution of purines and pyrimidines in all the nucleic acids studied renders the tetranucleotide hypothesis untenable.

5. The results of the analyses have indicated no great differences in the composition of these nucleic acids with respect to purines and pyrimidines.

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NOTE

A theoretical basis may be suggested for the ease of hydrolysis of the purine nucleosides and the great difficulty encountered with the corresponding pyrimidine derivatives.

The first step in the acid hydrolysis of both purine and pyrimidine nucleosides is an attack by hydronium ion at the glycosidic linkage. Specifically, a proton, leaving its coordination with water, may accept the free electrons of the nitrogen atoms in positions 9 of the purine ring and 3 of the pyrimidine ring (Fig. 5 A, B).

Whether this will occur to a significant extent depends on the energy level of the transition state. The proton, by accepting the free electrons of the nitrogen, forms an activated complex, or transition state, in which the ring carries a net positive charge. If the energy of formation of the charged intermediate is high the reaction is unlikely and the subsequent hydrolysis will not occur. If, however, the energy level of the charged intermediate is lowered by a resonance distribution of the charge throughout the ring, the frequency of its formation is correspondingly increased and so is the rate of hydrolysis.

In the addition of proton to the purine nucleosides the positive charge initially placed in the 9 position can be readily distributed by resonance throughout the imidazole and pyrimidine rings. Resonance structures in which the charge is shared by electronegative nitrogen may be expected to play a large part in the resonance stabilization of the transition state (Fig. 5 C). In guanosine, the tautomeric keto group in the 6 position resonates with an ionic form placing positive charge on the ring (Fig. 5 D). In the transition state this ionic form may be expected to hinder the introduction of an additional charge and minimize the resonance contribution of the pyrimidine ring. If this latter contribution were significant it would follow that guanosine, because of its keto group, should be somewhat more difficult to hydrolyze than adenosine, where the keto group is lacking. In the partial hydrolysis of the desoxypentose nucleic acid of calf thymus it was indeed found that adenine is released preferentially; *i.e.*, more rapidly than guanine. It should be emphasized, however, that the release of the bases from a polynucleotide may depend in large part upon other structural considerations.

All the naturally occurring pyrimidine nucleosides are characterized by the presence of one or more keto groups at the 2 and 6 positions of the ring. Ionic resonance of the 2 keto group (favored in acid media), by placing a positive charge adjacent to nitrogen 3, may be regarded as neutralizing its free electrons and leaving no point of attack for solvated proton (Fig. 5 E). The result is an *aromatic* resonance form in which nitrogen 3 bears a positive charge (Fig. 5 F). As long as such aromatic forms contribute a major share of the over-all resonance state of the molecule an attack by proton at position 3 is extremely unlikely. To overcome aromatic resonance stabilization and achieve the transition state high energies must be supplied. In keeping with theoretical predictions, the hydrolytic cleavage of pyrimidines from nucleic acids takes place only at elevated temperatures (120°) and in strongly acid solution. A further test of the theory is the prediction that once the aromatic resonance is destroyed (*e.g.* by reduction of the ring) the nitrogen in position 3 will retain a larger share of its free electrons and hydrolysis should proceed with comparative ease. The observation by Levene of the smooth

hydrolysis of dihydrouridine is in accord with this conclusion (Levene, P. A., and La Forge, F. B., *Ber. chem. Ges.*, 1912, **45**, 619).

To summarize, then, the facile acid hydrolysis of purine nucleosides is the result of resonance stabilization of the positive transition state. The pyrimidine nucleosides resist hydrolysis because the free electrons of nitrogen 3 are a part of the aromatic resonance structure of the ring and leave no effective point of attack for solvated proton. Formation of the transition state is possible only when high energies are supplied.

It should be emphasized that the hydrolytic release of the bases from a colloidal micelle may depend in large part on structural considerations not considered here.

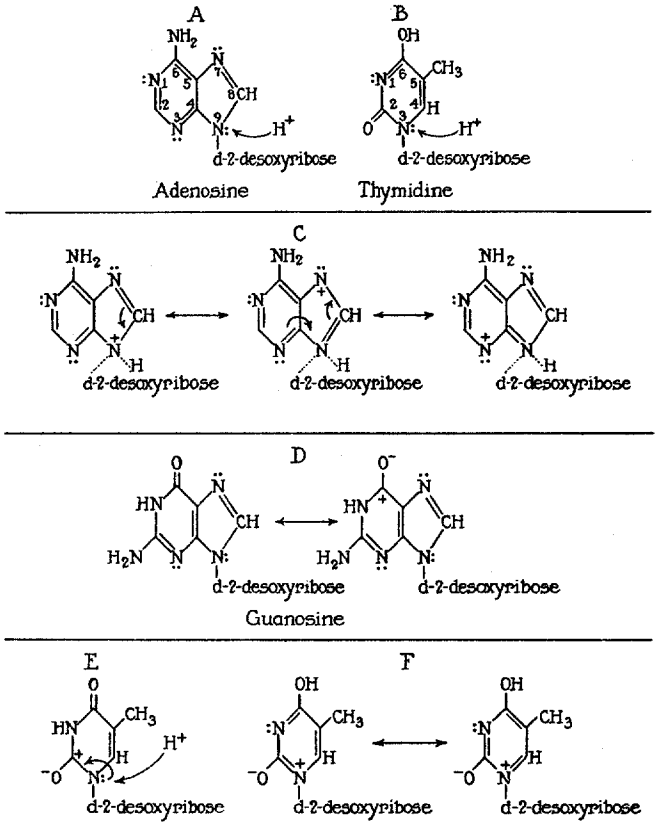

FIG. 5. Electronic resonance forms for purine and pyrimidine derivatives.