Chromatographic Studies on Nucleic Acids

2. THE QUANTITATIVE ANALYSIS OF RIBONUCLEIC ACIDS

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The nucleic acids are such universal constituents of biological systems and their function is probably so important that it is not surprising that much labour has been spent on the study of their composition. Until very recently there have been no methods of analysis which could be considered more than semi-quantitative, and it now seems doubtful whether many published analyses are even approximate. While it is now possible to separate and estimate quantitatively mixtures of purine and pyrimidine bases and some of their derivatives by means of paper strip chromatography (Hotchkiss, 1948; Vischer & Chargaff, 1948; Markham & Smith, 1949*b*), it is by no means as simple to convert nucleic acids into these compounds quantitatively. We have investigated many methods for the hydrolysis and estimation of nucleic acids, and have found it impossible to get satisfactory results by any published method. By combining our chromatographic technique with a simple method of quantitative hydrolysis and a solvent system which resolves the hydrolysate, we have evolved a new method of analysis which, in addition to being more sensitive than existing methods, is accurate and simple.

The hydrolysis of ribonucleic acids

Various methods have been used for the hydrolysis of ribonucleic acids. These depend upon the relative lability of the various linkages in these compounds to different treatments. For convenience they may be tabulated as follows: (1) Hydrolysis by NaOH or KOH at low temperature (20–37°), which was employed by Schmidt & Tannhauser (1945), liberates the nucleotides of the pyrimidine and purine bases. (2) Hydrolysis by NH_3 under pressure (Levene & Bass, 1931) is supposed to liberate nucleosides. (3) Hydrolysis by pyridine-water (Bredereck, Martini & Richter, 1941) is also said to liberate nucleosides. (4) Hydrolysis with weak mineral acid at 100°. This liberates the purines and the pyrimidine nucleotides. (5) Hydrolysis with mineral acids at high temperatures. This liberates both purine and pyrimidine bases. (6) Hydrolysis with formic acid at 175° (Vischer & Chargaff, 1948). (7) Enzymic methods. We have not investigated these.

Method (1) has been used, apparently with success, by Chargaff, Magasanik, Doniger & Vischer (1949). The four

nucleotides were not resolved on one chromatogram, so the solution was fractionated or a mixture of two nucleotides estimated from the absorption of the mixture at two wavelengths followed by the application of simultaneous equations. Using Whatman no. 1 paper and several mixtures designed to simulate as closely as possible the conditions described by these authors (isobutyric acid saturated with water, in the presence of NH_3 vapour), we were unable to obtain a satisfactory separation of an artificial mixture of uridylic and cytidylic acids.

Method (2) was used extensively by Levene and his colleagues. The actual temperature used was 145°, but as they invariably gave the temperature of the autoclave oil bath, it is commonly believed that a temperature of 175° was used for this hydrolysis (Levene & Jacobs, 1916). A certain proportion of the hydrolysate consists of nucleosides, but the method is not suitable for quantitative use as deamination takes place and some free bases are formed. For instance, adenosine treated by this method is partly converted to adenine, hypoxanthine and inosine.

Method (3), which has been used by Reichart (1948), involves refluxing with pyridine-water for 4–5 days and, besides being tedious, is not quantitative. Chromatograms of such a hydrolysate showed the presence of at least seven substances.

Method (4), hydrolysis with mineral acid, usually n-HCl at 100° for 1 hr., liberates the purine bases quantitatively. The pyrimidine nucleotides are also liberated, together with negligible amounts of the pyrimidine nucleosides. This is the method which was adopted and will be discussed at length later.

Method (5), hydrolysis with mineral acids at temperatures above 100°, liberates the purines and also free pyrimidines and some pyrimidine nucleosides. The purines are deaminated, and are eventually converted to glycine, formic acid, NH_3 and CO_2 (Krüger, 1892; Wulff, 1893). The cytosine is deaminated to uracil.

Hydrolysis with formic acid at 175° (method 6) liberates the bases with some loss of the purines and deamination. The cytosine is not deaminated to any extent, but for some reason the method does not give consistent or quantitative results for the pyrimidines, especially uracil. We have made many hydrolyses with formic acid for various periods of time in an unsuccessful attempt to make the method suitable for use with ribonucleic acids. It is an excellent method for the hydrolysis of uridine to uracil, giving quantitative recovery of uracil. In addition uracil added to thymus deoxyribose nucleic acid has been recovered quantitatively after hydrolysis.

EXPERIMENTAL

Chromatographic methods

Source of ultraviolet light. All chromatograms were run on Whatman no. 1 paper. The spots on the developed chromatograms were located in ultraviolet light (Markham & Smith, 1949*a, b*). Recently we have used as a source of ultraviolet light a Mazda 80 W. MB/V mercury lamp (British Thompson Houston) with the outer glass envelope removed. This lamp and the choke for controlling it are cheap. The lamp provides an intense source of ultraviolet light, which, though not as rich relatively in short-wave ultraviolet light as lower pressure mercury lamps, has a higher absolute intensity, thus allowing much shorter exposures and illuminating evenly a much greater area (an area of 12×14 in. of chromatogram being exposed for 15 sec. at a distance of 5 ft.). The filtered light from this lamp gives radiation predominantly of wavelength $265 \text{ m}\mu$, rather less of wavelength $253.7 \text{ m}\mu$ and a certain amount of general background.

Several people employing our method have used low-pressure mercury arc lamps (which emit mainly at $253.7 \text{ m}\mu$) without filters. While these give useful results, the definition is not as good as that obtained with a high-pressure arc used with filters.

Solvents. Having investigated the various hydrolysis procedures mentioned in the introduction we decided upon method (4), hydrolysis with *n*-HCl at 100° . The time chosen was 1 hr. as maximal yields of purines are given in this time. During the early stage of the work we employed as a routine a solvent containing 77% *n*-butanol, 13% water and 10% formic acid (v/v/v) (Markham & Smith, 1949*b*), which moves the purines, but leaves the nucleotides at the starting spot. It was desirable, however, to have a solvent which would move the nucleotides as well. From previous experience it was known that solvents containing more water than water-saturated *n*-butanol made the nucleotides move; on the other hand, it was essential to use an acidic solvent, as acid, besides accelerating the movement of the nucleotides, makes it possible to use relatively large quantities of guanine which is rather insoluble in neutral solutions or in the presence of NH_3 . Experiments with formic acid-propanol-water mixtures showed that the pyrimidine and purine nucleotides moved and increased in R_F with increasing acid concentration and also with increasing water content. *sec*.-Butanol-water mixtures were very similar, a mixture of 80% *sec*.-butanol, 20% water and 10% formic acid (v/v/v) proving incidentally a very useful solvent mixture for purines and pyrimidines. It was not possible, however, to get complete resolution in one dimension with formic acid mixtures, though this is easily possible in two dimensions. We therefore investigated other acids, both stronger and weaker than formic acid. HCl proved most suitable, and, eventually, by adjusting the water content and acidity of the solvent system, we arrived at a mixture consisting of 0.8*N*-HCl made up in a solution containing 70% by volume of *tert*.-butanol, the remainder of the mixture being water. To make this solution, take 700 ml. of pure *tert*.-butanol (at 26°), add 132 ml. of constant boiling HCl and make up to 1 l. at 20° . This solvent has several advantages. It separates guanine, adenine, cytidylic acid and uridylic acid in that order on a short strip of paper (Fig. 1), the R_F values being approximately 0.21, 0.32, 0.48 and 0.79 respectively, adequate resolution being given with

a total length of flow of 25 cm. The HCl front moves with the butanol front (in weak HCl solutions it lags behind) and the opaque brown line which coincides with the former lies quite clear of the uridylic acid spots. Being a very acidic solvent it is not necessary to neutralize the hydrolysate, the spots being put directly on to the papers, and in addition all the spots, including the guanine spot, are compact and show no tendency to tail. The only disadvantage of this solvent is that it runs rather slowly, a single run taking just under 2 days in battery-jar tanks ($14 \times 10 \times 35$ cm. high, internal dimensions) at $18\text{--}20^\circ$. It is necessary to dry the developed chromatograms carefully in a current of warm air without exposing them to high temperatures, which would cause the acid to attack the paper. Strips are hung with clothes pegs on a line, their lower ends being 20 cm. above a low-temperature 1 kW. electric radiator in a fume cupboard with the ventilating fan running. Drying takes 0.5–1 hr.

This solvent system is only suitable for the process for which it has been designed. It is not able to resolve the four nucleotides from yeast nucleic acid on a short strip of paper, and being very acid, is not recommended for use with labile substances; e.g. it hydrolyses yeast nucleic acid to nucleotides on the paper itself. Another point to note is that small changes in either the HCl or the water content of this solvent change the R_F values of the four substances in the hydrolysate and may make the resolution worse. On the other hand, the relative positions of these substances remain the same, even if the solvent composition varies over quite a large range. Generally it is unnecessary to run marker substances because of this, and, in any case, the absorption spectra of the substances in the spots are characteristic. It is also possible in case of doubt to elute a spot, evaporate it to dryness and subject it to further treatment. Cytidylic acid, for example, may be hydrolysed in formic acid by the method of Vischer & Chargaff (1948) and then be identified by running as a spot in *n*-butanol-formic acid or some other suitable solvent system.

Analytical methods

Phosphorus. After incineration of the sample with perchloric acid P was estimated as strychnine phosphomolybdate by Tisdall's (1922) colorimetric method.

Ribose. Purine-bound ribose was estimated by Militzer's (1946) orcinol method, as modified by Markham & Smith (1949*c*), using xylose as standard. Tests with the various ribosides showed that this modified method gives quantitative values for purine-nucleotide sugar, and does not give appreciable colour with that bound to pyrimidines.

Nitrogen. This was estimated by the usual micro-Kjeldahl method (Markham, 1942).

Biuret reaction. This was carried out using a drop of 0.5% CuSO_4 and KOH pellets in excess. Under these conditions a small quantity of ethanol added to the solution is salted out and takes all the biuret colour with it. Consequently the method is many times more sensitive than the usual one. (This technique, though discovered many years ago, does not seem to be at all well known. We have not been able to find the original reference.)

Hydrolysis of nucleic acid and estimation of purines and pyrimidine nucleotides

The nucleic acid was either evaporated to dryness in a current of air in a small evaporating tube immersed in a water bath at 100° or precipitated by dilute HCl and spun

down to the bottom of a small tube. N-HCl was then added to give a final concentration of the nucleic acid of about 10–20 mg./ml. The specific volume of nucleic acid was taken as about 0.6, the amount of N-HCl chosen accordingly; and 100 μl . of acid were usually quite sufficient. A small glass bead was added for mixing and the tube sealed and heated for 1 hr. at 100° in a water bath. It was cooled, opened, and 10–20 μl . quantities of the hydrolysate were measured out in capillary pipettes and placed on the filter paper about 6 cm. from the top. Two spots were placed on each sheet 9×34 cm., with 5 cm. between the spots for the blank strip. The paper was then developed in the *tert*.-butanol- HCl mixture for 2 days, dried and printed photographically. The fluorescence quenching technique of Holiday & Johnson (1949) may also be used, though we think that the spots are more obvious by our method. This is to be expected on account of the high contrast of the photographic materials. We find that guanine fluoresces quite brilliantly when excited by the 253.7 and 260 $\text{m}\mu$. light. The fluorescence effects may also be recorded photographically. If a thin sheet of cellulose nitrate is placed between the chromatogram and the reflex document paper, only the fluorescent light is able to reach the latter. The exposures are, of course, much longer. This technique could be used for recording the fluorescence of substances in 365–366 $\text{m}\mu$. light if a suitable filter were substituted for the cellulose nitrate film.

The spots on the chromatogram were cut out, together with the corresponding blanks, and each was extracted for at least 18 hr. in 5 ml. of 0.1 N-HCl in a test tube. The densities of the solutions were then read in a Beckman spectrophotometer, the following data being used:

Guanine	$D_{250} = 10.6$
Adenine	$D_{260} = 13.0$
Cytidylic acid	$D_{280} = 12.95$
Uridylic acid	$D_{260} = 9.89$

(The density D is for a cell 1 cm. thick, the subscript referring to the wavelength in $\text{m}\mu$. The concentration is 10^{-3} M. The values for cytidylic and uridylic acids are derived from the data of Ploeser & Loring (1949).)

Materials

Yeast nucleic acid. A commercial sample of about 2 g. of yeast nucleic acid was dissolved in 10 ml. of water by addition of the minimal amount of NH_3 and then precipitated by addition of 2 vol. of ethanol together with a few drops of N-HCl . The precipitate, collected by centrifugation, was redissolved in 10 ml. of water containing a small amount of NH_3 and the solution was cleared by centrifugation. This solution was shaken with 0.25 vol. of CHCl_3 and 0.1 vol. of amyl alcohol for 10 min. and the protein-containing chloroform-water gel removed (Sevag, Lackman & Smolens, 1938). This last procedure was repeated four times and was followed by dialysis against distilled water when some coloured material precipitated and was spun off. The nucleic acid was finally precipitated by acid ethanol, washed with 90% ethanol, 100% ethanol, ether, and then dried at 110° . The biuret reaction was negative.

After hydrolysis with 6 N-HCl at 120° for 6 hr. a fraction of the hydrolysed nucleic acid was chromatographed in *n*-butanol-acetic acid to detect amino-acids. The only amino-acid present other than in traces was glycine, which is, of course, derived from purines. The sugar was identified as

ribose by chromatography in *n*-butanol-water- NH_3 after hydrolysis of the nucleic acid in $\text{N-H}_2\text{SO}_4$.

Cytidylic acid, cytidine and uridine were obtained from Dr J. Davoll. Uridylic acid was a gift from Mrs L. D. Parsons and was precipitated twice as Pb uridylate and then as ammonium uridylate. Adenine and guanine were commercial preparations.

Fig. 1. Chromatogram of a hydrolysate of yeast nucleic acid. Yeast nucleic acid (YNA) was hydrolysed for 1 hr. at 100° in N-HCl . The hydrolysate was run on a chromatogram in 70% (v/v) *tert*.-butanol containing 0.8 N-HCl and water. The spots in descending order are: (a) guanine, (b) adenine, (c) cytidylic acid, (d) uridylic acid.

RESULTS

The specimen of yeast nucleic acid used was apparently free from all but traces of protein, and contained 8.4% phosphorus and 15.3% nitrogen. The

optical density of a 1 cm. thickness of a solution of this nucleic acid at a concentration of 49.2 $\mu\text{g./ml.}$ at pH 7.3 was 1.50 at 260 $\mu\text{m.}$ and 0.63 at 231 $\mu\text{m.}$ (at the maximum and the trough respectively). Solutions of this material, hydrolysed at 100° for 1 hr. in N-HCl and then chromatographed as described, gave rise to four major spots containing almost all the light-absorbing material which had been put on the filter paper. These spots (Fig. 1) are due to guanine, adenine, cytidylic acid and uridylic acid. The quantities of the purine bases and the pyrimidine nucleotides were estimated spectrophotometrically in the extracts of the spots. The chromatographing and estimation of this sample of nucleic acid were done in quadruplicate and the results, together with other relevant analytical data are given in Table 1.

Table 1. *Composition of yeast nucleic acid by weight*

Constituent	$\mu\text{g./mg.}$ dry wt.	$\mu\text{mol./mg.}$
Adenine	101	0.746
Guanine	137	0.906
Cytosine	63.7	0.574
Uracil	75.4	0.674
Purines	238	1.65
'Purine-bound' ribose	239	1.59
Total bases	377	2.90
Phosphorus	84	2.71
Nitrogen	153	10.9
Nitrogen calculated from bases	151	10.8
Nucleic acid calculated from bases on basis of simple poly- nucleotide structure	942	—

The four spots account for 98.3 % of the nitrogen present in the nucleic acid, the remainder presumably being accounted for by nitrogenous impurities in the nucleic acid and slight loss of pyrimidine nucleotide during the hydrolysis. On a dry-weight basis 94.2 % of the material is accounted for as free nucleic acid, some of the residual 5.8 % probably being due to base combined with the acid. The standard errors of the molar ratios of the four bases were computed in the usual way, assuming that the errors are distributed randomly and these are presented in Table 2 with the corresponding molar

Table 2. *Composition of yeast nucleic acid.*
Ratios of the four bases with standard errors

Base	Molar ratio (mean = 1)
Adenine	1.03 $\pm$ 0.01
Guanine	1.25 $\pm$ 0.01
Cytosine	0.80 $\pm$ 0.02
Uracil	0.93 $\pm$ 0.04

ratios. The errors obtained are of similar magnitude to those found in the analyses of other ribonucleic acids by this method. The sources of error due to various causes may be assessed by inspection of the amounts of the substances recovered from individual

spots put on the papers, and these suggest that the errors are not due entirely to pipetting.

DISCUSSION

While previously published methods tested by us have failed to give consistent results in the analysis of ribonucleic acids, this method has been found reliable and simple. The method of hydrolysis used is extremely mild and consequently the purines are not destroyed, while the pyrimidine nucleotides liberated are not hydrolysed further to an appreciable extent. The nucleic acid specimen used was apparently remarkably pure, its analysis comparing favourably with those of other purified yeast nucleic acids. The fact that over 98 % of the nitrogen, and virtually all of the light-absorbing material could be accounted for by the sum of the pyrimidine nucleotides and the purine bases indicates that the hydrolysis is quantitative. It will be noted in this regard that the sum of the bases, made up as it is from four estimates all having measurable standard errors, is itself subject to a standard error of about $\pm 1.2\%$. This point is frequently disregarded in making up balance sheets of the composition of complex biological substances.

The value for free ribose, as estimated colorimetrically (Table 3), agrees within reasonable limits of error with the quantity which one would expect to find associated with the purines. The figure for phosphorus, on the other hand, is slightly lower than one would expect on the basis of a simple polynucleotide structure requiring one atom of phosphorus per molecule of base. We are inclined to

Table 3. *Composition of yeast nucleic acid.*
Molar ratios of constituents

(The theoretical values are for a simple polynucleotide.)

	Found	Theory
Purine/pentose	1.04	1.00
Bases/phosphorus	1.67	1.00
Base N/total N	0.98	1.00
Purines/pyrimidines	1.32	—

attribute this to small systematic errors in the analyses rather than to postulate a branched structure for yeast nucleic acid, though the latter has been suggested by Gulland (1947). While no further evidence as to the nature of the internucleotide linkage has been obtained, it is certainly remarkably labile, and any attempt to isolate dinucleotides or small polynucleotides will necessitate novel methods of hydrolysis and estimation.

In common with other nucleic acids which we have investigated, this sample of yeast nucleic acid has failed to give equimolar proportions of the bases (Table 2). This is not surprising since the methods used in the original investigations which suggested

this ratio were very crude by modern standards. What is more surprising is that this nucleic acid approximates so closely to a tetranucleotide, the maximum discrepancy being only 20 %. It is evident, however, that, if all the nucleic acid particles in the preparation have a similar composition, the minimum 'molecular weight' of a polynucleotide must be at least 100,000. The inference is that the particles, as they exist in commercial yeast nucleic acid, are not all alike.

SUMMARY

A simple chromatographic method for the quantitative analysis of the bases of ribonucleic acids is described. The amount necessary for one analysis is about 0.1 mg. One hydrolysis only is needed and all the bases are recovered from single spots after chromatography on filter paper. Analyses of a specimen of yeast nucleic acid are given.

REFERENCES

- Bredereck, H., Martini, A. & Richter, F. (1941). *Ber. dtsch. chem. Ges.* **74**B, 694.
 Chargaff, E., Magasanik, B., Doniger, R. & Vischer, E. (1949). *J. Amer. chem. Soc.* **71**, 1513.
 Gulland, J. M. (1947). *Sym. Soc. exp. Biol.* **1**, 8.
 Holiday, E. R. & Johnson, E. A. (1949). *Nature, Lond.*, **163**, 250.
 Hotchkiss, R. D. (1948). *J. biol. Chem.* **175**, 315.
 Krüger, M. (1892). *Hoppe-Seyl. Z.* **16**, 160.
 Levene, P. A. & Bass, L. W. (1931). *Nucleic Acids*. New York: The Chemical Catalog Co. Inc.
 Levene, P. A. & Jacobs, W. A. (1916). *J. biol. Chem.* **25**, 103.
 Markham, R. (1942). *Biochem. J.* **36**, 790.
 Markham, R. & Smith, J. D. (1949a). *Nature, Lond.*, **163**, 250.
 Markham, R. & Smith, J. D. (1949b). *Biochem. J.* **45**, 294.
 Markham, R. & Smith, K. M. (1949c). *Parasitology*, **39**, 330.
 Militzer, W. E. (1946). *Arch. Biochem.* **9**, 85.
 Ploeser, J. McT. & Loring, H. S. (1949). *J. biol. Chem.* **178**, 431.
 Reichart, P. (1948). *Nature, Lond.*, **162**, 662.
 Schmidt, G. & Tannhauser, S. J. (1945). *J. biol. Chem.* **161**, 83.
 Sevag, M. G., Lackman, D. B. & Smolens, J. (1938). *J. biol. Chem.* **124**, 425.
 Tisdall, F. F. (1922). *J. biol. Chem.* **50**, 329.
 Vischer, E. & Chargaff, E. (1948). *J. biol. Chem.* **176**, 715.
 Wulff, C. (1893). *Hoppe-Seyl. Z.* **17**, 468.

Chromatographic Studies on Nucleic Acids

3. THE NUCLEIC ACIDS OF FIVE STRAINS OF TOBACCO MOSAIC VIRUS

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The tobacco mosaic virus was the first of several plant viruses to be shown to be nucleoprotein by the work of Bawden, Pirie, Bernal & Fankuchen (1936) and that of Bawden & Pirie (1937). The importance of the nucleic acid, although originally disputed by workers in America, is now accepted as a matter of course, and it seems probable that nucleic acid is an essential factor for the multiplication of many viruses. In their original work Bawden & Pirie (1937) isolated the nucleic acid in several ways and showed that it resembled the ribose nucleic acid of yeast. Later Loring (1939) showed that its constituents were at least similar to those in yeast nucleic acid. Recently we have developed an accurate quantitative method for the analysis of ribonucleic acids (Smith & Markham, 1950), and it seemed desirable to see whether the virus nucleic acids resembled yeast nucleic acid in their quantitative composition, and to what extent, if at all, the

various viruses differ in the make-up of their nucleic acids. In this paper we discuss the results obtained with five different strains of virus, all of which have serological groupings in common and all of which are similar morphologically.

EXPERIMENTAL

Materials

The viruses used in this investigation were all related strains belonging to the tobacco mosaic virus group. All were passed through single lesions on *Nicotiana glutinosa* (excepting cucumber virus 4 which does not go to this plant) in order to avoid any possibility of strain mixtures. The purification method used followed the lines of that of Bawden & Pirie (1937).

The strains used were as follows:

Tobacco mosaic. The ordinary type strain, which has been maintained here for many years, has from time to time been put through single lesions to maintain its purity. It does