Chromatographic Studies of Nucleic Acids

4. THE NUCLEIC ACID OF THE TURNIP YELLOW MOSAIC VIRUS, INCLUDING A NOTE ON THE NUCLEIC ACID OF THE TOMATO BUSHY STUNT VIRUS

BY R. MARKHAM AND J. D. SMITH

Agricultural Research Council Plant Virus Research Unit, Molteno Institute, University of Cambridge

(Received 23 November 1950)

It was largely because of our interest in the turnip yellow mosaic virus (Markham & Smith, K. M., 1949) that we began our study of nucleic acids. This virus contains about 37 % by weight of ribonucleic acid, which is an unusually high proportion. All the infected plants which we have examined contain, as well as the virus, another characteristic and related protein in an amount equivalent to 25–30 % of the weight of the virus nucleoprotein. This protein, as far as we know, cannot be separated from the virus by any method other than fractional centrifuging, as the two proteins differ very little, if at all, in their solubilities in various solutions, in electrical charge and in their size, shape and general surface properties. This second protein appears to be the virus nucleoprotein minus its nucleic acid, and it is non-infectious. It is a very poor antigen in rabbits, although it combines readily with antiserum to the nucleoprotein, which itself is an excellent antigen. In these two properties, ability to cause a disease and ability to cause antibody production in rabbits, we would appear to have biological activities associated with a nucleic acid, and with a view to obtaining further information about the function of nucleic acid in viruses we have undertaken the analysis of several virus nucleic acids.

We describe in the following paper several refinements of our earlier techniques and we have confirmed most of our findings by orthodox micro-analytical procedures, which, combined with chromatographic methods, can be used to analyse ribonucleic acids without the need for an ultraviolet spectrophotometer.

METHODS AND MATERIALS

Precautions necessary for maximum accuracy

When we began our investigation of nucleic acids it seemed that an error of $\pm 5\%$ or thereabouts could be tolerated, and such a figure may be obtained without a great deal of trouble by the method which we have already published (Smith, J. D. & Markham, 1950). However, as we checked the various sources of variability in our methods, it became evident that considerably greater accuracy was possible, and as small variations in the compositions of the nucleic acids of plant viruses may be of importance, we considered it worth while to improve our methods.

Much of the inaccuracy when dealing with small quantities of materials is due to systematic volumetric errors, and in this investigation we have used throughout one capillary pipette having a volume of $14.0\ \mu\text{l.}$, both for measuring out the solutions to be put on the chromatograms and for taking samples for P, N and sugar analysis. This simple precaution eliminates most of the systematic error.

It is obvious that extreme care should be taken in calibrating the cells used for spectrophotometry before making each set of readings, and, although tedious, it is a good plan to read all the solutions including the blanks in one cell against a water blank in the other cell. A considerable source of error was traced to the test tubes used for extracting the spots. Traces of commercial detergent give rise to measurable densities in the ultraviolet and are difficult to remove completely. In consequence we have cleaned all test tubes used with a few drops of ethanol followed by conc. HNO_3 , rinsing after the violent reaction with tap water and then distilled water.

The last main limiting factor in the spectrophotometric estimation is the paper blank reading which is generally between 0.01 and 0.05 density units, increasing from the top of the sheet to the solvent front. This variation may be made negligible by the use of large quantities of nucleic acid hydrolysate, and it is in this that chromatographic solvents containing strong HCl show their superiority, as they can move 1–2 mg. of nucleic acid hydrolysate without any sign of 'tailing'. There would, however, appear to be no advantage in increasing the quantity of nucleic acid in each spot above 1.5 mg. as this amount gives densities of at least 1.0–2.0 in the individual extracts in 5 ml. of 0.1 N-HCl. It is very striking that when replicate spots in this density range are read they usually do not differ by more than ± 0.002 unit. When working in this range, the correction for the volume occupied by the nucleic acid in solution becomes important if the results have to be referred to a dry weight basis, being of the order of 5–10%. The correction may, of course, be determined experimentally by measuring the P content of the hydrolysate, but the approximate correction which we have given previously should be adequate for most purposes. The minimum amount of fluid for the hydrolysis should be 0.05 ml., as below this level the volume change due to the saturation of the air space in the hydrolysis tube may become limiting.

With the refinements given above, the overall accuracy is such that it becomes necessary to take into account the hydrolysis of the pyrimidine nucleotides. We have determined the extent of this hydrolysis experimentally as being of the order of 5%, and this correction may be applied without determining it for each hydrolysis.

Preparation of virus nucleic acid

The nucleic acid of the turnip yellow mosaic virus is liberated from the latter by treatment with 30% or more (v/v) of ethanol in neutral solution at room temperature (Markham & Smith, K. M., 1949). Several preparations have been made by this method from crystalline virus. Subsequent treatment involved removal of proteins by centrifuging, and by the method of Sevag, Lackman & Smolens (1938). The material was used in an 'air-dry' condition and probably contained a considerable amount of water. Tests for protein were negative.

Analytical methods

P was estimated throughout by Allen's (1940) method. Total P and the P bound to pyrimidines were estimated by incinerating with 60% (w/v) HClO_4 , while inorganic P was estimated without further hydrolysis, either from pipetted volumes of liquid or on extracts of filter-paper spots made in dil. HClO_4 . The incineration of spots cut from developed chromatograms proceeds fairly smoothly, and we have only had a small number of the incinerations explode. It is, however, wise to take the precaution of wearing some protection for the eyes when near the digestions. The blank spots cut out as controls were estimated at about 0.5 μg . P so that quantities of some 25–50 μg . of bound P could be estimated with fair accuracy. In the chromatograms run in solvent 3 (see below) the blanks tended to be rather higher and more variable than with the other solvents.

Inorganic P was estimated from spots on the chromatogram by finding its position by the method of Hanes & Isherwood (1950) and then cutting out duplicate spots from parallel lanes. The spots were then extracted overnight in 10 ml. of water, washed out and made up to volume with the reagents used for the P estimation.

The absorbing spots on the chromatogram were found by our usual method (Markham & Smith, J. D., 1949) in some cases with a modified filter system (Markham & Smith, J. D., Appendix). We have also used the fluorescence method of Holiday & Johnson (1949) (see also Carter, 1950), viewing the chromatograms through a violet filter (e.g. Wratten C_5). But we have found it impossible in some cases to see any guanylic acid, apparently because the fluorescence of the latter matched that of the filter paper.

The other analytical procedures used have been described previously (Smith, J. D. & Markham, 1950). The molecular extinction coefficient for guanine which we now use is slightly different from that used previously ($\epsilon \times 10^{-3}$ at 250 m μ . in 0.1 N-HCl = 11.0). This value, obtained by Wyatt (1951) and confirmed by us, is 4% higher than our earlier figure.

Hydrolysis methods

Two methods of hydrolysis have been used. Method (i), using N-HCl at 100° for 1 hr. (Smith & Markham, 1950), liberates adenine, guanine, cytidylic acid, uridylic acid and small amounts of cytidine and uridine (about 5% of the pyrimidine nucleotides are hydrolysed to nucleosides).

Method (ii) is hydrolysis with N-NaOH or N-HCl at room temperature, which yields a solution containing the nucleotides of the four bases. N-NaOH is the better of the two as ribonucleic acid becomes sticky in N-HCl and it takes a long time for solution to take place. As a routine we place the nucleic acid in a small serum bottle, add the NaOH and leave 18 hr. or more before using. No further hydrolysis

takes place so that the solution may be kept for weeks. The solution is put on to the papers and if acid solvents are used need not be neutralized. On the other hand, with solvent 3 it is necessary to neutralize the spot on the paper with a small excess of N-acetic acid and to dry before running the chromatogram. If N-HCl is used for the hydrolysis, it is important that the spot should not be allowed to dry, as in this process the acid increases in strength and further hydrolysis of the nucleotides may take place.

Chromatographic solvents

For chromatography of the mixtures obtained by hydrolysing the nucleic acid in several ways we have used three solvents. Solvent 1 is 7.45 M-*tert*.-butanol, 0.8 N-HCl in water (Smith, J. D. & Markham, 1950). Solvent 2 is 8.5 M-*iso*-propanol, 2 N-HCl in water (Wyatt, 1950). These two mixtures are similar in their ability to resolve the various bases, but differ in that no. 1 resolves uridine while no. 2 resolves cytidine, thus permitting the estimation of the traces of these nucleosides which are present in hydrolysate (i).

As in the case of most chromatographic solvents consistent R_F values may only be obtained with these mixtures by taking elaborate precautions such as maintaining constant temperatures and exact maintenance of solvent composition, etc. The relative positions of the substances, however, are reasonably constant and it is possible to identify most spots on chromatograms by inspection. Consequently we have been in the habit of using hydrolysates of nucleic acids of known composition as markers, as such markers give much more information than spots of single substances. Fig. 1 gives an indication of the positions of the spots on chromatograms run in these solvents. Only uridylic acid is resolved in either solvent from the mixture of nucleotides produced by cold acid or alkaline hydrolysis (Fig. 1 B).

Solvent mixture 3, which we developed for separating the purine nucleotides from the mixture produced by cold alkaline hydrolysis, differs greatly from solvents 1 and 2. It consists of saturated $(\text{NH}_4)_2\text{SO}_4$, 79%, water, 19%, and *iso*propanol, 2% (v/v/v). A buffer solution (0.1 M, pH about 6) may with advantage be substituted for the water, but it is not essential. It may also be found advantageous to have a small excess of *iso*propanol in the solvent in the bottom of the tank. This solvent runs rapidly, 6 hr. being the usual time for running 30 cm.

Commercial yeast adenylic acid gives two spots when run in this solvent, the one with the larger R_F value (adenylic acid *a*) running slightly behind muscle adenylic acid and comprising about 25.6% of the total (this value is only a characteristic of the method of production of this adenylic acid). From this it would appear that adenylic acid *a* is the same as the adenylic acid *a* of Carter (1950), who originally found that yeast adenylic acid was a mixture of two related substances. The more slowly moving substance, constituting 74.4% of this specimen of commercially purified yeast adenylic acid, separated in solvent mixture 3, presumably corresponds with

Carter's adenylic acid *b*. In hydrolysates of ribonucleic acids these two adenylic acids are also found, and in addition two guanylic acids (also named *b* and *a* in order of increasing R_F value) are resolved on the chromatogram while the uridylic and cytidylic acids

RESULTS

For the purposes of comparison we have converted all the analytical results into $\mu\text{mol./mg.}$ dry weight of the starting material. This procedure has the advantage that all the values may be compared

Fig. 1. Chromatograms of hydrolysates of ribonucleic acids. *A*, hydrolysed in N-HCl , 1 hr. at 100° run in solvent 1; *B*, hydrolysed in N-NaOH at 20° run in solvent 2; *C*, hydrolysed in N-HCl , 1 hr. at 100° run in solvent 3. The spots are: 1, guanine; 2, adenine; 3, cytidylic acid; 4, uridylic acid; 5, H_3PO_4 ; 6, cytidine; 7, uridine, and 8, a mixture of adenylic, guanylic and cytidylic acids.

are not resolved from each other (Fig. 2). A disadvantage of this solvent is that it is unable to move large quantities of the nucleotides and so it is necessary to spread out the hydrolysate of 1 mg. of a nucleic acid over 5 cm. at the top of the filter paper instead of putting it on as a compact spot.

Fig. 2. Relative positions of nucleotides in solvent 3. From top to bottom: $\square$, adenylic acids *b* and *a*; ▨ , guanylic acids *b* and *a*, and $\blacksquare$, cytidylic + uridylic acid.

directly. It is surprising that this method of presentation is not adopted more generally in making up balance sheets of complex substances such as proteins.

Table 1. *Composition of turnip yellow mosaic virus nucleic acids as determined by ultraviolet light-absorption measurements*

(The two sets of figures refer to analyses from the nucleic acids of two entirely different virus preparations. The figure in brackets is the number of sets of determinations.)

Substance	Preparation 1 (4) ($\mu\text{mol./mg.}$)	Preparation 2 (3) ($\mu\text{mol./mg.}$)
Adenine	0.616	0.60
Guanine	0.460	0.44
Total purines	1.08	1.04
Cytidine	0.06 ($\pm 1.8\%$)	—
Cytidylic acid	0.98	1.01
Total cytosine	1.04	1.06
Uridine	0.02	—
Uridylic acid	0.59	0.58
Total uracil	0.61	0.61*
Total pyrimidines	1.65	1.67
Total bases	2.73	2.71
Weight of nucleotide residues	866 $\mu\text{g./mg.}$	

* Determined on alkaline hydrolysate.

Table 2. *Composition of turnip yellow mosaic virus nucleic acids with their standard errors as determined by normal micro-analytical techniques*

Substance	$\mu\text{mol./mg.}$	Substance estimated	No. of estimations
Total purines	1.07	Pentose	2
Total purines	1.03 ($\pm 2\%$)	Inorganic P*	6
Uridylic acid	0.58 ($\pm 1.6\%$)	Uridylic acid P†	3
Total pyrimidines	1.67 ($\pm 1.7\%$)	Total pyrimidine P	3
Cytidylic acid	1.09	By difference	—
Cytidylic acid	1.07 ($\pm 1.4\%$)	Cytidylic acid P†	5
Total bases	2.72	Total P	4
Total bases accounted for by purine P + pyrimidine P	2.70	—	—

* 5% of the total pyrimidine P subtracted. † Value found $\times 1.05$.

Table 3. *The ratios of the nucleotides in turnip yellow mosaic virus nucleic acid calculated to an arbitrary total of 4.00*

Substance	Molar ratio from absorption	Molar ratio from N estimation
Adenylic acid <i>a</i>	0.51*	—
Adenylic acid <i>b</i>	0.40*	—
Total adenylic acid	0.91	0.88
Guanylic acid <i>a</i>	0.35*	—
Guanylic acid <i>b</i>	0.34*	—
Total guanylic acid	0.69	0.75
Cytidylic acid	1.53	1.46
Uridylic acid	0.89	0.91
Purines/pyrimidines	0.65	—

* Obtained from the ratios of the two extinctions and the total purine found.

Hydrolysates prepared from turnip yellow mosaic virus nucleic acid were analysed in the following way. Adenine, guanine, cytidine, cytidylic acid and uridylic acid were estimated spectrophotometrically after chromatography of the hydrolysate in solvent 2. Uridine in the hydrolysate was estimated after chromatography in solvent 1. The results are presented in Table 1. The standard errors were only calculated for cytidine as the maximum difference observed in the measurements of the purines and the pyrimidine nucleotides was 0.7%. The total of the bases accounts for 102% of the phosphorus in the nucleic acid if one assumes one atom of phosphorus per molecule of base. This value is not significantly greater than the expected 100%.

The following substances were estimated:

(1) Total P was estimated on samples of hydrolysates (i) and (ii).

(2) Uridylic acid P from spots of hydrolysate (ii) run in solvent 2. This analysis gives the total uridylic acid P, as in this hydrolysis no uridine is formed.

(3) Cytidylic acid + uridylic acid P was also estimated in the same hydrolysate from spots run in solvent 3, and hence by subtraction the cytidylic acid P was determined.

(4) Cytidylic acid P was estimated in hydrolysate (i) using solvent 2. This value may be corrected to a close approximation to give the total cytidylic acid P (including that originally associated with the cytidine) by multiplying by 1.05, a value deduced from the relative absorption of these substances when extracted from the chromatogram.

(5) The phosphorus associated with the purines + the pyrimidine nucleosides was estimated on the whole hydrolysate (i) and also on the spots of phosphoric acid from chromatograms run in solvent 2. The results in both cases were identical. Using a minor correction for the P liberated from pyrimidine nucleotides (approx. 5% of the total pyrimidine) the purine P may be deduced.

(6) Pentose was estimated in hydrolysate (ii). The sugar was identified as ribose by a chro-

matographic method (Markham & Smith, J. D., 1950).

These results are tabulated in Table 2. The molar ratios of the nucleotides calculated to an arbitrary total of 4 are given in Table 3. These values are derived from the figures in Table 1.

Nitrogen estimations were also made on spots cut out from chromatograms of an acid hydrolysate run in solvent 2. The results showed considerable variation because of the small amounts of purine or pyrimidine nitrogen in the spots (22–53 $\mu\text{g.}$) relative to the blank in the paper (about 20–40 $\mu\text{g.}$), but as a matter of interest are given in Table 3 for comparison. The total recovery of nitrogen from the spots was equivalent to 2.64 $\mu\text{mol.}$ of base per mg. nucleic acid, but is, of course, subject to considerable error.

The purine nucleotides

Values are given in Table 3 for the relative amounts of the two isomeric adenylic and guanylic acids which we found in chromatogram spots of hydrolysates run in solvent 3. These spots have been identified by extracting and hydrolysing in *N*-hydrochloric acid at 100° for 1 hr. and running in solvent 1

Fig. 3. The ultraviolet absorption spectrum of turnip yellow mosaic virus nucleic acid. A solution equivalent to 4.82 $\mu\text{g. P/ml.}$ in a 1 cm. cell.

or 2. That these double spots are not artifacts may be demonstrated by cutting them out and re-running in solvent 3, when they maintain their relative positions even after further treatment with *N*-sodium hydroxide. This indicates that they are not an equilibrium mixture formed in alkaline solution, although we have confirmed Doherty's (1950) observation that they are interconvertible in acid solution. While we have not been able to make accurate P analyses on these spots, our values are sufficiently reliable to show that one atom of P is combined with each molecule of purine in these nucleotides. The absorption spectrum of the whole nucleic acid is shown in Fig. 3.

The nucleic acid of the tomato bushy stunt virus

The nucleic acid of this virus has also been analysed, but the method used has been slightly different. The nucleic acid is more difficult to remove from the protein, so we hydrolysed the whole virus.

Virus (4 mg.) was taken in a small test tube in the form of a solution. The virus was precipitated by adding $(\text{NH}_4)_2\text{SO}_4$ to 1/3 saturation and was centrifuged down. The supernatant liquid was poured off and the tube wiped inside with filter paper. *N*-HCl (0.05 ml.) was added and the stoppered tube was placed in a water bath at 100° for 1 hr. Most of the protein was insoluble after this treatment and was sedimented by centrifuging. The supernatant liquid was pipetted off on to the filter paper for chromatography. Five 14 $\mu\text{l.}$ spots were run in solvent 2, three of which were then used for spectrophotometric determination of the purines and the pyrimidine nucleotides.

The results are given in Table 4. The other two lanes of the chromatogram were neutralized with ammonia vapour and then sprayed with ninhydrin to check that no aromatic amino-acids were present to interfere. The amount of free amino-acids was very small.

Table 4. Molar ratios of the bases of tomato bushy stunt virus nucleic acid, calculated to an arbitrary total of 4.00

Adenine	0.995
Guanine	1.12
Cytosine	0.875
Uracil	1.01
Purines/pyrimidines	1.12

Standard errors were computed from the readings of the densities and were small. The largest of the series was uridylic acid, $\pm 1.5\%$, which is quite satisfactory in view of the small amount of material used.

DISCUSSION

The figures presented in Tables 1–3 show that it is possible by using certain precautions to analyse a ribonucleic acid with considerable accuracy either by ultraviolet absorption measurements on chromatogram spots or by phosphorus analyses on similar spots in conjunction with other measurements. The former is obviously the method of choice, but it is apparent that in order to take full advantage of the reproducibility of the technique it is necessary to apply minor corrections for the hydrolysis of the pyrimidine nucleotides during the liberation of the purines. As the correction is small and fairly constant, the most convenient method of calculating the results is to use the following molar extinction coefficients:

Guanine	$\epsilon_{250} = 11.0 \times 10^3$
Adenine	$\epsilon_{260} = 13.0 \times 10^3$
Cytidylic acid	$\epsilon_{280} = 12.3 \times 10^3$
Uridylic acid	$\epsilon_{260} = 9.45 \times 10^3$

The dash signifies that the extinction coefficient is an apparent and not an actual one. Using this set of figures and taking the precautions outlined it should be possible to obtain results within about $\pm 1\%$ on any ribonucleic acid. In this regard it should be pointed out that in cases such as that of

Fig. 4. The compositions of four ribonucleic acids (N.A.) in molar proportions of nucleotides. From left to right turnip yellow mosaic N.A., tomato bushy stunt N.A., tomato mosaic N.A. (Markham & Smith, J. D., 1950) and yeast N.A. (Smith, J. D. & Markham, 1950). The columns are: □, adenylc acid; ▨, guanylic acid; ■, cytidylic acid, and ▩, uridylic acid. (These conventions are also observed where possible in Figs. 1 and 2.)

the turnip yellow mosaic virus nucleic acid, where large amounts of cytidylic acid are found, it is advantageous to use solvent 2, as the error which may be introduced into the estimation of cytidylic acid by the presence of uridine is very small when readings are made at $280\text{ m}\mu$, while the absorption of cytidine may cause an appreciable if small error in the adenine estimation. Solvent 2, however, needs to be run at least 30 cm. down the paper, but this is to some extent compensated for by its greater rate of flow.

A fact which emerges from the present analyses of the nucleic acids of turnip yellow mosaic virus and the tomato bushy stunt virus and the previous analyses which we have made, is that the general pattern of these ribonucleic acids is very different (Fig. 4). While it is evident that more analyses of different plant viruses need to be made, the results suggest that the ribonucleic acids may be characteristic of virus 'genera' and thus be useful in the classification of viruses. It is with this point in view that we have started to develop methods of analysis which can be applied to small quantities of whole virus, enabling data to be obtained for viruses which can only be prepared in milligram quantities.

We have given in this paper data concerning the amounts of the isomeric adenylc and guanylic acids in turnip yellow mosaic virus nucleic acid, but feel that their interpretation must await further work. If their properties are found to be characteristic they might prove to be of considerable importance in the study of nucleic acid structure, but if, as seems likely, they are only α and β forms of the nucleoside-3-phosphoric acids as has been suggested recently (Doherty, 1950), it might be dangerous to stress the significance of differences.

SUMMARY

1. Improvement in methods for the chromatographic analysis of ribonucleic acids are described. The overall accuracy is of the order of $\pm 1\%$.
2. A method for the quantitative analysis of ribonucleic acids by chromatography combined with normal micro-analytical techniques is outlined.
3. Turnip yellow mosaic virus nucleic acid has been analysed by both methods and close agreement has been obtained between the results.
4. Tomato bushy stunt virus nucleic acid has been analysed by a modified technique suitable for application to small quantities of virus material.

REFERENCES

- Allen, R. J. L. (1940). *Biochem. J.* **34**, 858.
 Carter, C. E. (1950). *J. Amer. chem. Soc.* **72**, 1466.
 Doherty, D. G. (1950). *Proc. Amer. chem. Soc.*, 3 September 1950, 56C.
 Hanes, C. S. & Isherwood, F. A. (1950). *Nature, Lond.*, **164**, 1107.
 Holiday, E. R. & Johnson, E. A. (1949). *Nature, Lond.*, **163**, 216.
 Markham, R. & Smith, J. D. (1949). *Biochem. J.* **45**, 294.
 Markham, R. & Smith, J. D. (1950). *Biochem. J.* **46**, 513.
 Markham, R. & Smith, K. M. (1949). *Parasitology*, **39**, 330.
 Sevag, M. G., Lackman, D. B. & Smolens, J. (1938). *J. biol. Chem.* **124**, 425.
 Smith, J. D. & Markham, R. (1950). *Biochem. J.* **46**, 509.
 Wyatt, G. R. (1950). *Nature, Lond.*, **166**, 237.
 Wyatt, G. R. (1951). *Biochem. J.* **48**, 584.