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The Ribonucleic Acid Content of Turnip Yellow Mosaic Virus*

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ABSTRACT: The ribonucleic acid (RNA) content of turnip yellow mosaic virus (TYMV) was derived from an experimentally determined ratio of N:P (21 determinations), and the theoretical nitrogen and phosphorus contents of TYMV–RNA and TYMV–protein which were calculated from the respective base ratio and amino acid composition. The ratio of N:P was 4.64 (σ 0.14), the content of RNA (in the acid form) 33.5%. The nitrogen and phosphorus contents of TYMV were subsequently calculated to be 15.19 and 3.27%, respectively. By relating ultraviolet absorbancy at 260 mμ and the individual nitrogen and phosphorus determinations to each other, and adjusting the latter to the theoretical values, an extinction coefficient of $E_{260}^{1\%} = 86$ was obtained for TYMV. The data are consistent with an optical determination of the RNA content, with a number of recently determined physical constants of TYMV and its constituent components, and with the notion that its capsid is an icosahedral arrangement of 180 protein subunits of 20,000 mol wt.

The detailed structural analysis of viruses, consisting predominantly of nucleic acid and protein, requires an exact knowledge of the absolute amounts in which these components occur in a virus particle. Such knowledge is provided by the determination of the nucleic acid content (usually by chemical means) and of the particle weight (usually by physicochemical methods) of the virus.

Turnip yellow mosaic virus,[1] a small isometric plant virus, has been known to contain a relatively large amount of nucleic acid since it was first purified, although the initial estimates were too low because of the presence of top component particles or empty capsids in the preparations (Markham and Smith,

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[1] Abbreviations used: TYMV, turnip yellow mosaic virus; RNA, ribonucleic acid.

1949). When at a later stage of its study the infectious nucleoprotein particles were separated from the top component particles (Markham, 1951) and the nucleic acid was identified to be of the ribose type (RNA) (Markham and Smith, 1950, 1952), more accurate estimates of the actual RNA content could be made (Markham, 1959). Nonetheless, at the time the present study was begun, there seemed to exist considerable disagreement in the literature as to the exact RNA content of TYMV. Values of 34% (Cosentino *et al.*, 1956), 36% (Matthews, 1960), 37% (Markham, 1959; Mitra, 1964), 38% (Matthews, 1958), and 39% (Haselkorn, 1962) have been reported.

If, in addition, the equally wide range of published particle weights of TYMV (5.0–5.8 million) is taken into consideration, it is clear that investigators concerned with the precise analysis of the particle's substructure could face profound disagreement with others who happen to use a different set of basic constants for the virus (see, *e.g.*, Klug and Finch, 1960; Harris and Hindley, 1961, 1965; Markham *et al.*, 1963).

While the problem of the particle weight of TYMV will be treated in separate communications (W. Godschalk, G. Mayer, and H. Familant; J. M. Kaper, W. Godschalk, F. N. Weber, and D. W. Krupke, in preparation), this publication deals with the systematic estimation of its RNA content in a great number of carefully purified preparations. It starts from the premise that a great deal of the present confusion could very well have originated in an apparent lack of systematic approach to the problem, and possibly in certain errors in analytical technique, among which the problem of obtaining highly accurate dry weights stands out in particular. The latter problem can be circumvented, however, thanks to the fact that nowadays, in contrast to the situation with the more fundamental properties mentioned above, there is available in the literature a set of highly reproducible data concerning the base composition of TYMV–RNA (Markham and Smith, 1950; Symons *et al.*, 1963; Rauws *et al.*, 1964) and the amino acid composition of TYMV–protein (Harris and Hindley, 1961, 1965; Symons *et al.*, 1963). In addition, it is known that the virus contains 0.7% of its weight of the polyamine bis(3-aminopropyl)amine (Johnson and Markham, 1962). These data allow for the precise calculation of the phosphorus and nitrogen contents of the respective components, which, in combination with an accurately determined ratio of N:P in TYMV, lead directly to its content of ribonucleic acid.

Materials and Apparatus

All chemicals were obtained commercially and were of analytical quality. Virus was prepared from infected Chinese cabbage plants that were cultivated in a growth room under artificial illumination and at constant temperature. The isolation and purification procedure employed was that described by Steere (1956).

Centrifugation for preparative purposes was performed with the Spinco Model L ultracentrifuge; for analytical purposes, the Spinco Model E ultracentrifuge equipped with Schlieren optics was employed.

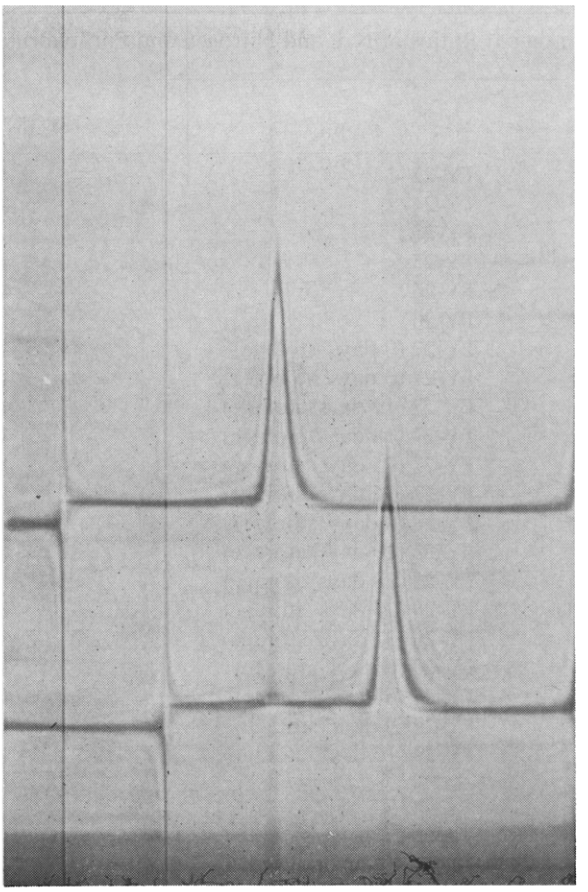

FIGURE 1: Sedimentation patterns of TYMV. Sedimentation from left to right. Picture taken at 50° schlieren angle, 16 min after centrifuge attained speed of 23,150 rpm; temperature, 20°. Lower pattern: stock preparation of TYMV at 0.3% concentration with slight contamination of top component. Upper pattern: same, after removal of top component.

Ultraviolet absorption measurements were performed with the Cary Model 14 recording spectrophotometer; the Beckman DU spectrophotometer was used in colorimetric phosphorus determinations.

Methods

Preparation of Virus for Analyses. Stock preparations of TYMV obtained by differential centrifugation of butanol–chloroform treated juice from infected plants usually still contain slight traces of contaminating top component particles. Figure 1, lower pattern, shows the sedimentation pattern of a stock preparation of TYMV. The simplest (although rather wasteful) method to eliminate these traces of top component is to continue with a few cycles of short duration ultracentrifugation (30 min at 40,000 rpm) until no top component can any more be detected in the sedimentation diagrams of the

TABLE I: Ratios of N:P and Nitrogen and Phosphorus Contents of Individual TYMV Preparations.

Prepn	Ratio N:P	Nitrogen Content[a] (%)	Phosphorus Content[a] (%)
TY-22	4.67	15.81	3.38
TY-24	4.67	15.67	3.35
TY-25	4.48	14.76	3.30
TY-25	4.71	15.00	3.19
TY-26	4.81	15.59	3.24
TY-26	4.32	14.09	3.26
TY-27 (0 days' storage)	4.62	15.45	3.34
TY-27 (6 days' storage)	4.82	16.03	3.32
TY-27 (12 days' storage)	4.67	15.67	3.35
TY-27 (18 days' storage)	4.52	14.94	3.30
TY-27 (25 days' storage)	4.77	15.52	3.25
TY-27 (32 days' storage)	4.31	14.89	3.45
TY-28 (0 days' storage)	4.73	15.03	3.18
TY-28 (10 days' storage)	4.64	14.67	3.16
TY-28 (18 days' storage)	4.67	14.77	3.16
TY-28 (24 days' storage)	4.63	15.15	3.27
TY-28 (31 days' storage)	4.62	14.96	3.24
TY-29 (0 days' storage)	4.71	15.56	3.30
TY-29 (6 days' storage)	4.63	15.29	3.30
TY-29 (9 days' storage)	4.76	15.60	3.27
TY-29 (16 days' storage)	4.74	14.97	3.16
	Av: 4.64 (σ 0.14)	Av: 15.21 (σ 0.47)	Av: 3.28 (σ 0.08)

[a] Experimental data adjusted to $E_{260}^{1\%}$ (TYMV) = 86.

resuspended virus pellets (Figure 1, upper pattern). Such preparations were stored in 0.01 M KH_2PO_4–NaOH buffer of pH 7 in the cold with a few drops of chloroform as a preservative, until used. At time intervals of several days, aliquots containing sufficient virus to determine an ultraviolet spectrum, and to perform nitrogen and phosphorus analyses, were withdrawn and subsequently exhaustively dialyzed *vs.* glass-distilled water in the cold. The virus solution then underwent a short centrifugation at 10,000 rpm to remove small amounts, if any, of sediment that occasionally developed during storage, and was then suitably diluted for the series of determinations mentioned above.

Determination of Concentration. Virus concentration was estimated by means of its ultraviolet absorption spectrum determined in triplicate. An extinction coefficient $E_{260}^{1\%}$ = 85 obtained from preliminary studies (Kaper, 1964) was applied.

Determination of Total Nitrogen and Phosphorus. Nitrogen determinations were performed by means of the micro-Kjeldahl method. Virus samples containing *ca.* 400 μg of nitrogen were digested with concentrated H_2SO_4 and a catalyst consisting of a mixture of equal parts of K_2SO_4, $CuSO_4$, $HgSO_4$, and Se. Intermittent addition of 30% H_2O_2 aided the oxidation. After addition of excess NaOH, the alkaline digests were steam distilled and the ammonia-containing distillate was trapped in a boric acid solution. Subsequently, the solution was titrated with 0.01 N HCl, using methyl red as an indicator. Each nitrogen determination was run in triplicate, the figures in Table I representing their averages. Ammonium sulfate of known concentration, run simultaneously, was used as standard.

Phosphorus was determined essentially as described by Knight and Woody (1958). Each virus sample, usually containing 5–10 μg of phosphorus, was determined in quadruplicate and the determined phosphorus contents averaged. In each determination, two known standards of KH_2PO_4 were run simultaneously.

Alkaline Hydrolysis of TYMV and Optical Determinations. TYMV was hydrolyzed with 0.5 N NaOH at 37° for 24 hr. Measured aliquots were subsequently neutralized with 1.0 N HCl and diluted with a large excess of 0.1 M sodium phosphate buffer of pH 7. The absorbancy at 260 mμ was read *vs.* 0.1 M sodium phosphate buffer of pH 7.

Results

RNA Content from N:P Ratios. Table I presents the results of the nitrogen and phosphorus determinations in their entirety. Altogether 21 ratios of N:P were determined in seven different batches of purified TYMV. In some preparations only one or two analyses were

performed (TY-22, 24, 25, and 26); others, however, were analyzed several times after the preparations had been allowed to age for periods of up to 1 month (TY-27, 28, 29). This is approximately the maximal lifetime of TYMV preparations used for other investigations in our laboratory. Since no systematic changes could be detected in the N:P ratios or in the individual nitrogen and phosphorus contents with increasing time of storage of the preparations, it was decided to base the final ratio of N:P on the average of these determinations individually instead of taking it as the average of the N:P ratios of the separate virus preparations (each of the virus preparations would carry a different statistical weight). The averaged final ratio of N:P and its standard deviation are listed at the bottom of column 2 in Table I.

For the purpose of calculating the RNA content of TYMV, each particle was considered to consist of two components, RNA (as the undissociated acid) and the protein plus polyamine combined. The mole fractions of these components will be represented by X and $(1 - X)$, respectively. The individual nitrogen and phosphorus contents of TYMV–RNA were calculated to be 15.51 and 9.76%, respectively, using the base ratio of the type strain (Symons *et al.*, 1963). The nitrogen content of the protein plus polyamine combined was calculated from the amino acid analyses of the type strain (Symons *et al.*, 1963) and the data of Johnson and Markham (1962) on the amount of polyamine per virus particle. This nitrogen content turned out to be 15.03%. The mole fraction of RNA in TYMV was subsequently calculated by solving the following equation for X

$$\text{N:P determined} = \frac{(X \times \%\text{ N in RNA}) + ((1 - X) \times \%\text{ N in protein + polyamine})}{(X \times \%\text{ P in RNA}) + ((1 - X) \times \%\text{ P in protein + polyamine})}$$

$$4.64 = \frac{(X \times 15.51) + (1 - X) \times 15.03}{(X \times 9.76) + 0}$$

$$X = 0.335$$

Therefore, the RNA content of TYMV (taking RNA in its acid form) is $100 \times X = 33.5\%$. With the help of this figure, and with the calculated nitrogen and phosphorus contents of the individual components, the theoretical nitrogen and phosphorus percentages of an individual TYMV particle can be calculated

$$\%\text{ N in TYMV} = (0.335 \times 15.51) + (0.665 \times 15.03) = 15.19\%^{2}$$

$$\%\text{ P in TYMV} = (0.335 \times 9.76) = 3.27\%$$

[2] Recently, Kupke has carefully related dry weight of TYMV to its nitrogen content and found 15.18% for the latter, showing a remarkable agreement with the theoretical value (D. W. Kupke, personal communication).

Specific Extinction Coefficient of TYMV. The above theoretical percentages of nitrogen and phosphorus and the averages of the determined values (which were based on an extinction coefficient $E_{260}^{1\%} = 85$) were used to readjust the extinction coefficient to its true value. This was then found to be $E_{260}^{1\%} = 86$. The individually determined percentages of nitrogen and phosphorus were subsequently readjusted, using the true virus concentrations, and are presented in columns 3 and 4 of Table I. Their final averages and standard deviations are given at the bottom of the respective columns.

RNA Content from Optical Determinations. When a known amount of virus is hydrolyzed in alkali and its absorbancy determined after neutralization to pH 7, the latter is a direct measure of the absolute amount of RNA in the sample provided that proper corrections are applied for the contribution of protein absorbance at 260 mμ. Table II presents the results of four such determinations of the RNA content in two different virus preparations. The average nucleic acid content obtained was 33.7%.

Discussion

The work described above represents an attempt to bring clarity in a matter which for some time has contributed to different interpretations of the precise substructure of TYMV. With this determination of the RNA content of TYMV, which we now believe to be 33.5%, it is for the first time that a figure was obtained which is internally consistent with many other basic data determined simultaneously, but, more importantly, which is also in agreement with the detailed aspects of the model proposed for TYMV on the basis of electron-microscope and X-ray diffraction studies (Huxley and Zubay, 1960; Nixon and Gibbs, 1960; Klug and Finch, 1960; Klug *et al.*, 1965). According to the latter, TYMV-protein is an icosahedral shell built up of 180 basic peptide chains with a molecular weight of 20,000 (Harris and Hindley, 1961, 1965; Symons *et al.*, 1963). From this it follows that the capsid's particle weight should be 3.6 million [consistent with an earlier determined value of 3.5 million (Kaper, 1960)]. Using the presently determined RNA percentage of the virus, it follows that its molecular weight is 5.4 million, a figure in good agreement with the equilibrium studies on the magnetic ultracentrifuge (Godschalk *et al.*, and J. M. Kaper *et al.*, in preparation). A further inference is that the molecular weight of TYMV-RNA is 1.9 million, which also agrees reasonably with more recent estimates from sedimentation viscosity studies (Mitra, 1964) and from light scattering measurements of the isolated RNA (Hirth *et al.*, 1965). Furthermore, the present estimate can be compared with the RNA content calculated from recent careful determinations of the partial specific volumes of TYMV, top component, and isolated TYMV–RNA by means of the magnetic densitometer (Ulrich *et al.*, 1964), assuming additivity of these constants on the basis of the gross composition of TYMV. The partial specific volumes of the above materials were estimated to be: $\bar{V}_{\text{TYMV}} = 0.661$, $\bar{V}_{\text{TC}} = 0.734$, and $\bar{V}_{\text{RNA}}$

TABLE II: Optical Determinations of the RNA Content of TYMV.

Prepn	Before Hydrolysis Absorbancy[a] (260 mμ)	Concn[b] of TYMV (μg/ml)	After Hydrolysis: Absorbancy[a] of TYMV (260 mμ)	After Hydrolysis: Absorbancy[c] of RNA (260 mμ)	After Hydrolysis: Concn[d] of RNA (μg/ml)	RNA Content (%)
TY-18	0.271	31.4	0.355	0.341	10.3	33.0
TY-18	0.260	30.2	0.358	0.3445	10.4	34.6
TY-29	0.222	25.8	0.300	0.2885	8.75	34.0
TY-29	0.188	21.7	0.250	0.240	7.3	33.3
						Av: 33.7%

[a] Each absorbancy is given as the average of at least a triplicate optical determination. [b] Using $E^{1\%}_{260}$ (TYMV) = 86. [c] Obtained from previous column after substraction of protein absorbancy at 260 mμ which was calculated from the known amounts of phenylalanine, tyrosine, and tryptophan (Symons *et al.*, 1963) and their respective ultraviolet spectra (Wetlaufer, 1962). [d] Using $E^{1\%}_{260}$, pH 7 (TYMV-RNA hydrolyzed) = 330 as calculated from nucleotide molar extinction coefficients (Beaven *et al.*, 1955) and base composition data (Symons *et al.*, 1963).

= 0.509 (Ulrich and Kupke, 1964; D. V. Ulrich, J. M. Kaper, and D. W. Kupke, in preparation). From these values, it can be calculated that TYMV should contain 32.6% RNA, which is in reasonable agreement with the data presented in this paper, and at least confirms the trend toward a lower RNA content than had generally been accepted for TYMV so far.

A careful survey of the literature data revealed that the RNA content, as determined by Cosentino *et al.* (1956), by means of pentose and phosphorus analyses approaches the presently estimated value the closest. The value of this confirmation is doubtful, however, in view of the uncertainty of the pentose analyses as mentioned by the authors. Furthermore, the assumption of 16% nitrogen in TYMV is incorrect, as can be seen from the calculated nitrogen contents of TYMV-protein and TYMV-RNA in the Results section. Finally, in this publication, an extinction coefficient of $E^{1\%}_{260}$ = 101 is given for TYMV, which is inconsistent with the low RNA content reported (see later in this discussion). Only one report could be found in which a relatively low extinction coefficient was determined for TYMV (Horn *et al.*, 1963). These authors give $E^{1\%}_{260}$ = 84 and mention a reproducible phosphorus content of 3.15–3.30% for the virus, both results in remarkable agreement with ours. However, in their paper they fail to draw the conclusion that such a phosphorus content is indicative of an RNA content of *ca.* 33% if combined with the calculated phosphorus content of 9.76% for TYMV-RNA.

Mitra (1964) determined an RNA content of 37% for TYMV, using an optical method similar to the one employed in this study (Table II). This estimate was not entirely independent, however, since the extinction coefficient of TYMV used was taken from the literature (Haselkorn, 1962) (see later in this discussion). If $E^{1\%}_{260}$ = 86, as determined in this study, is used with Mitra's experimental data, an RNA content close to 35% can be calculated.

Matthews (1960) has reported a mean RNA content of 35.6% for the B_1 fraction of TYMV preparations fractionated by means of CsCl density-gradient centrifugation. This figure was derived from an experimentally determined ratio of N:P (seven determinations) and an assumed phosphorus content of 9% for TYMV-RNA, while the nitrogen contents of both RNA and protein were taken to be 15%. (It should be noted that this is basically the same method that was employed in the present study.) Taking these figures, it can be calculated that Matthews' average N:P ratio must have been 4.68, which is extremely close to our determined N:P of 4.64. Using the calculated nitrogen and phosphorus contents, as given in the Results section, an N:P of 4.68 would have led to an RNA content of 33.1%. In an earlier publication, however, Matthews (1958) correlated dry weight, absorbancy at 260 mμ, and phosphorus content of TYMV preparations containing different amounts of top component. While the correlation of phosphorus content with an absorbancy level of 0.86 (see Figure 1 in Matthews, 1958) is in reasonable agreement with the specific extinction coefficient of 86 and 3.27% phosphorus found in this work, Matthews found 3.53% of phosphorus in a purely nucleoprotein preparation. This led to the conclusion that TYMV contained 38% RNA (as the ammonium salt) or 36% as the free acid. The reason for the above inconsistency is difficult to assess.

Likewise, Markham (1959) reported an extinction coefficient of 95, an RNA content of 37%, but a phosphorus content of 3.77% which, on its own, yields an RNA content of 38.8% when combined with the calculated phosphorus content of the RNA. Haselkorn (1962), however, in a report from the same laboratory, mentioned a redetermination of the RNA content and the extinction coefficient of TYMV and found 39% and 89 (at 262 mμ), respectively, but no further details were given.

A critical appraisal of our experimental data in rela-

tion to the other studies led us to the conclusion that, to our knowledge, the present work has been the most extensive and systematic study thus far performed on the RNA content of TYMV. While certainly not entirely independent because of the use of calculated phosphorus and nitrogen contents of the components of TYMV, this method has the decided advantage of by-passing the problem of accurate determinations of dry weight, often a source of analytical error. As was pointed out in the introduction, the foundations of this work (TYMV's base ratio and amino acid composition) seem remarkably well established. However, should these data have to be revised in the future, it will now require just a simple calculation to revise the RNA content concomitantly. The analytical error in the N:P analyses seems within the limits that can reasonably be expected. The only suitable yardstick with which to compare our results was the determination of the phosphorus content of tobacco mosaic virus (Knight and Woody, 1958). In this careful study, 70 separate determinations were performed on 19 different batches of virus. We have calculated that the standard deviation in these 70 determinations was 2% of the average, the standard deviation found in our work (σ = 0.08 = 2.4%) being of the same order of magnitude. Similarly, the average nitrogen content had a standard deviation of σ = 0.47 = 3.1%, while the average N:P had σ = 0.14 = 3.0%. If the latter is incorporated in the calculations of the RNA content, the upper and lower limits of this value become 34.6 and 32.6%, respectively. A further confirmation of the results derived from the N:P determinations was obtained in the optical studies (Table II), although admittedly, like in Mitra's work (Mitra, 1964), these were not entirely independent because of the use of $E_{260}^{1\%}$ = 86 for TYMV, a figure derived from the nitrogen and/or phosphorus *vs.* absorbancy correlations.

As the above literature survey has shown, there appears to be more agreement with the results reached in the present study on the RNA content of TYMV than is suspected at first glance. If a rough borderline of agreement and disagreement is drawn at 35% RNA, then only the values of Matthews in one of his two reports (Matthews, 1958), Markham (1959), and Haselkorn (1962) seem to deviate appreciably. In view of the results and the discussion presented above, we feel justified to propose an RNA content of 33.5% for TYMV.

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