Reactivity of Tobacco Mosaic Virus and Its Protein toward Acetic Anhydride*

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ABSTRACT: Treatment of glycyltyrosine with acetic anhydride at various pH values suggests that the unprotonated forms of the amino and phenolic groups represent the two reactive species. Thus no marked selectivity of the reagent for ϵ-amino over phenolic groups would be expected in proteins, were it not for the fact that in native proteins the amino groups are frequently located externally and the phenolic groups internally. In tobacco mosaic virus (TMV) protein, the two lysine residues (residues 53 and 68) and two of the four tyrosine residues become readily acetylated, one of the tyrosines exceeding the lysines in rate of acetylation. In the intact TMV particle, one tyrosine and one of the lysines (residue number 68) react preferentially, though slower than in the isolated protein. This differential reactivity is discussed in the light of other studies which indicated that the residues 64–67 of the peptide chain are comparatively peripheral on the virus particle, and that the C-terminal part (138–158) is particularly accessible. Reconstitution of virus is possible only with those acetylated protein preparations which carry acetyl groups at residues which are acetylatable in the intact virus (lysine 68 and one tyrosine, presumably tyrosine 139).

The amino acid sequence of the protein of tobacco mosaic virus (TMV) is known to favor a specific folding of the peptide chain so that upon denaturation the protein appears able to exactly reform the secondary, tertiary, and quaternary structure of the native protein and the virus rod (Anderer, 1959). The nature of side-chain interactions underlying these reactions is of obvious interest. The selective modification of various protein groups represents one approach to the identification of these interactions. Previous papers have dealt with the role of the one SH and the four tyrosine groups (Fraenkel-Conrat, 1955, 1958; Fraenkel-Conrat and Sherwood, 1967). The present study was initiated in the hope that acetic anhydride would supply a means of selectively acetylating one or both of the amino groups in TMV protein. However, it soon became evident that this reagent showed no such specificity when acting on this protein or on the intact virus. Actually, the most reactive group in TMV and TMV protein appeared to be a phenolic one. Nevertheless, the lability of the *O*-acetyl bond has made it possible to differentiate *O*- and *N*-acetylation. Acetylation of the intact virus has given further indications regarding the spatial arrangement of the peptide chains in the virus (Colloms, 1966a,b).

Methods and Materials

[^{14}C]Acetic anhydride was obtained from New England Nuclear Corp. (1–5 mc/mmole). It was usually diluted with [^{12}C]acetic anhydride to about 300,000 dpm/μmole and stored over $CaCl_2$ in a refrigerator. It decomposed to about 50% in 1 year. To check the accuracy of dilution the acetyl derivatives of valine and arginine were prepared, purified by paper or Dowex 50-X4 chromatography, and their specific amino acid content as determined by means of the Spinco amino acid analyzer was related to the counts per minutes determined in a Packard scintillation spectrophotometer. Frequently the acetic anhydride was for convenience's sake diluted with benzene (1:10). Such dilutions did not appreciably decrease the anhydride content over a period of several days, nor did the amounts of benzene added affect the ultraviolet absorbancy of the protein solutions at 278 mμ to a detectable extent.

O-Acetyl groups were usually determined by ultraviolet spectrophotometry. The absorbancy of the protein (1 ml) at 278 mμ was determined, then 0.1 ml was withdrawn, 0.1 ml of 1 M NaOH was added, and the absorbancy was again determined. Both absorbancies were corrected for buffer absorption and light scattering ($A_{278\ a}$) and ($A_{278\ b}$). The latter ($A_{278\ b}$) was also corrected for dilution and shift in spectrum by a factor of 1.133 to obtain $A_{278\ c}$. The difference between this and the original absorbancy ($A_{278\ c} - A_{278\ a}$) divided by the difference in the 278-mμ absorbance of tyrosine and *O*-acetyltyrosine (1.160) yields micromoles of OAc per milliliter. The concentration of protein (micromoles per milliliter) in the sample is derived from the corrected absorbancy in alkali ($A_{278\ c}$) by dividing by 22.0.

Amino groups were determined by means of ninhy-

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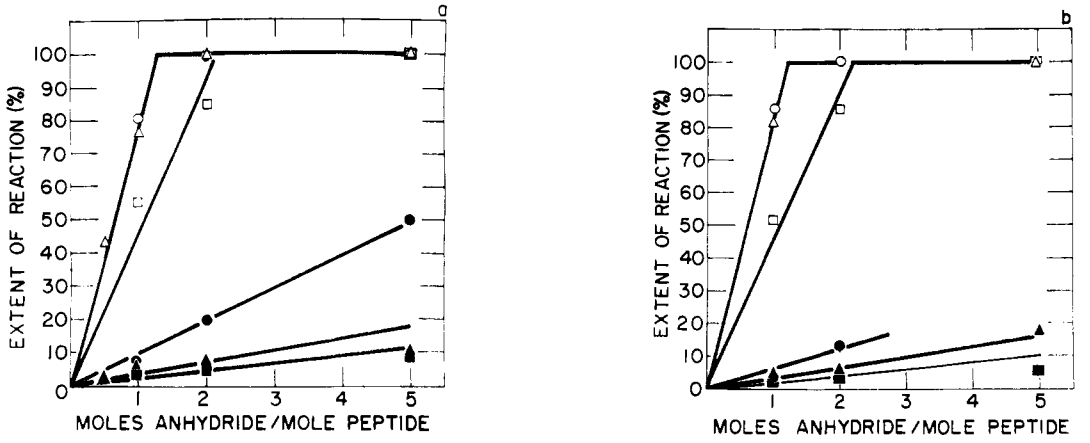

FIGURE 1: Effect of pH on specificity of acetylation of glycyl-L-tyrosine. Extent of amino reaction was determined from the remaining ninhydrin color. The extent of phenolic group acetylation was obtained from the decrease in extinction coefficient of the peptide at 278 mμ. Open symbols represent amino group acetylation. Closed symbols represent phenolic group acetylation. (a) Phosphate buffer concentration, 0.5 M; peptide concentration, 5.0 mM; pH 6.0 (□), pH 6.6 (△), and pH 7.5 (○). (b) Phosphate buffer concentration, 0.05 M; peptide concentration, 0.5 mM; pH 6.2 (□), pH 6.6 (△), and pH 7.6 (○).

drin according to Cocking and Yemm (1957), preevaporating the samples with 0.1 M $NaHCO_3$ to remove ammonia. Sodium acetate (4 M) was used, and frequently guanidine-HCl was added to 2 M to the 60% ethanolic diluant to avoid turbidity. Sulfhydryl was determined by titration (Fraenkel-Conrat, 1957a).

Results

Acetylation of Tyrosine and Glycyl-L-tyrosine. Experiments to test the specificity of the action of acetic anhydride were performed with tyrosine and particularly with glycyl-L-tyrosine, as exemplified on Table I and Figure 1. These data showed that a distinct preference for amino acetylation was attained in the range of pH 6.2–7 although the reaction rate was lower the lower the pH. The pH dependence suggested that the unprotonated amino groups and the dissociated phenolic groups represented the reactive species.

TABLE I: Acetylation of Glycyl-L-tyrosine.

Buffer and pH	Extent of Acetylation[a]		
	O-Ac (%)	*N*-Ac (%)	*N*-Ac/*O*-Ac
Sodium citrate (0.45 M), 5.5–5.4	4	66	17
Potassium phosphate (0.45 M), 6.7–6.5	13	100	8
Half-saturated sodium acetate, 8.6–6.9	75	100	1.3

[a] A 20-fold excess of acetic anhydride was used. The extent of *O*-acetylation was determined spectrophotometrically and the *N*-acetylation by the amino acid analyzer (15-cm column), the unacetylated peptide emerging at about 44 min compared to 37 min for tyrosine.

The nature of the buffer appeared not to play a critical role and acetate buffer did not favor selectivity for the amino group. The fact that greater specificity could be attained with glyclytyrosine than with tyrosine was attributed to the greater differential in the pK values of the NH_2 and OH group for the dipeptide.

The conclusion that the pK of the amino group was a decisive factor differentiated these model experiments from the situation prevailing in TMV protein where only ϵ- rather than α-amino groups were to be acetylated. These model experiments thus made it appear unlikely that acetic anhydride would show marked specificity for ϵ-amino as contrasted to phenolic groups if both types of groups were similarly located in terms of steric accessibility.

Acetylation of TMV Protein. As exemplified on Table II, acetylation with varying amounts of acetic anhydride has always yielded preparations containing partly labile (*O*-acetyl) and partly stable (*N*-acetyl) radioactivity. The labile acetyl groups were released by treating the protein with 0.1 N alkali or with 1 M hydroxylamine at pH 9.

The determination of total bound acetyl derived from its ^{14}C content generally agreed with the *N*-acetyl and *O*-acetyl as derived from ninhydrin analysis and spectrophotometric analysis, respectively. These data were also frequently confirmed by densitometry of polyacrylamide gel electropherograms, on the one hand (see later), and loss in radioactivity on the other. Thus neither the SH group nor other protein groups appear to

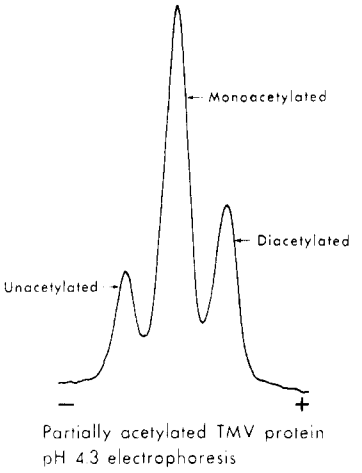

FIGURE 2: Microdensitometer tracing of polyacrylamide electrophoresis gel. Migration of sample at pH 4.3 was toward cathode (−). Optical density units for the ordinate are arbitrary in the densitometry trace.

bind acetyl groups to a detectable extent. Direct SH analyses confirmed this, since the SH titer was between 80 and 91% of that of the unacetylated protein (1.0/mole).

It was evident from these data that the lysine amino groups, and part of the tyrosine phenolic groups of TMV proteins reacted simultaneously with acetic anhydride at pH 6.3–7.7. Maximally the two lysines and two of the four tyrosines became acetylated under these conditions.

The data on Table II further suggested that one tyrosine was more reactive than the two lysines, and the other less. The selectivity of the reagent was not improved by changes in pH, protein concentration, or other variables. High concentrations of acetate had no favorable effect during or after the reaction, in contrast to the findings reported by Riordan *et al.* (1965).

The question as to the relative reactivity of the two lysines was investigated by determining the proportion of mono- and di-*N*-acetylated proteins in partially acetylated reaction mixtures. Polyacrylamide gel electrophoresis presented a convenient tool for this purpose. Thus electrophoresis of a preparation containing one *N*-acetyl group per mole showed the presence of the three possible components: unacetylated, mono-, and diacetylated proteins. In amounts, as measured by the areas on densitometry plots, these accounted for 16, 56, and 29% of the total (Figure 2). These studies thus showed that the introduction of the first acetyl group did not represent a rate-limiting step, since in that case the partially acetylated preparation would have contained predominantly unacetylated and diacetylated protein.

The definitive allocation of the stable acetyl to the lysine residue was done after digestion of the diacetylated protein with trypsin and fractionation of the resultant peptides. Peptide 3 which contains an internal lysine not susceptible to trypsin was found to be replaced by an acetylated peptide and peptides 4 and 5 were missing, and replaced by a fused acetyl derivative as expected if the acetylation of lysine 68 rendered this peptide bond resistent to trypsin (Figure 3). Peptide 3, as well as its acetyl derivative, was frequently dual, both upon chromatography and electrophoresis, a fact which is attributed to the N terminus of this peptide being partly in the pyroglutamyl form. Upon electrophoresis at pH 9.2, however, this peptide moved as a single component, and this technique has facilitated the separation and quantitation of the two acetyl peptides (3 and 4 plus 5 (Figure 4)). When this method was used with partially acetylated preparations, it definitely indicated that there was little, if any, difference in the reactivities of the two lysines in TMV protein toward acetic anhydride, since similar amounts of label were found associated with lysines 53 and 68. The observed ratios of acetylation of 68:53, were usually between 0.85 and 1.5, and averaged 1.1 for preparations containing a wide range of acetyl groups (0.2–2.0).

Acetylation of TMV. The reaction of the virus with acetic anhydride was considerably less efficient, and thus presumably slower than that of the isolated protein

TABLE II: Acetylation of TMV Protein.

Conditions of Acetylation[a]		Extent of Acetylation[b]	
pH	Acetic Anhydride[c]	*O*-Ac[c]	*N*-Ac[c]
6.3	15	0.4	0.2
6.6	15	1.1	0.6
7.1	15	1.7	1.9
7.4	15	2.0	2.1
7.7	15	2.1	2.2
7.6	9	1.7	1.0
	17	1.9	1.5
7.5[d]	44	1.2	0.4
9.5[d]	44	1.1	1.1
Half-saturated acetate	6 (48 hr)[e]	1.2	0.4
	30 (30 hr)[e]	1.2	0.5
	60 (0.25 hr)[e]	1.2	0.7

[a] In 0.02 M potassium phosphate in a pH-Stat at 0°; the pH dropped occasionally by up to 0.2 unit. The acetic anhydride was in most cases added as a 1:10 solution in benzene. [b] *O*-Acetylation was determined spectrophotometrically (see Methods). *N*-Acetylation was an average of data obtained from various measurements (radioactivity after alkali treatment or hydroxylamine, ninhydrin color, etc). [c] Moles per mole of proteins. [d] These experiments were performed at unusually low protein concentration (0.03 mM, 0.02 M phosphate) in the spectrophotometer cell (25°), and *O*-acetylation was determined directly. [e] Acetylated protein held in acetate buffer for times indicated in parentheses before isolation and analysis.

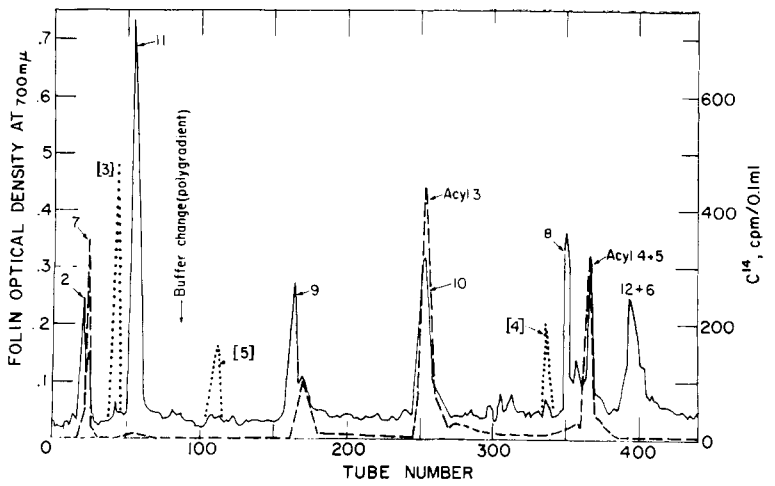

FIGURE 3: Dowex 1-X2 chromatographic separation of soluble fraction of tryptic digest of acetylated TMV protein. The column chromatography was carried out as described by Funatsu (1964) on the peptides soluble at pH 4.5 after tryptic digestion. The fractions were 3.3 ml each. The numbers refer to the sequential location of the main component of the peaks in the whole TMV protein chain. Peptide 1 is the insoluble peptide removed by precipitation at pH 4.5. (———) Folin color; $OD_{700\ m\mu}$/0.2-ml aliquot of alternate tubes; (- - - -) radioactivity (cpm/0.1 ml); (· · · ·) typical location of missing peptides.

at the same pH. A 25-fold reagent excess, sufficient to completely acetylate the protein, introduced only about one stable and one labile acetyl group into the virus, and six times as much reagent was needed to achieve maximal acetylation.

When the protein was isolated from acetylated virus, by the acetic acid method (Fraenkel-Conrat, 1957b) an analysis of the nature of the diacetyl derivative by the methods described above showed that the one *N*-acetyl group introduced into the virus was not, as in the case of protein acetylation, randomly distributed over lysines 53 and 68, but was predominantly located on lysine 68 (ratio of 68:53 acetylation = 3.4). Thus it appeared that lysine 68 was considerably more reactive toward acetic anhydride in the intact virus, than residue 53.

Ability of Acetylated Protein to Reconstitute Virus. Experiments were performed to test the ability of various acetylated preparations of TMV protein to form infective virus with added TMV-RNA under the usual reaction conditions (Fraenkel-Conrat and Singer, 1964). Preparations containing only one *N*-acetyl group reconstituted to about 5 and 21% without and with prior removal of the 1.7 *O*-acetyl groups by hydroxylamine treatment. However, preparations containing more than one acetylated lysine became progressively more insoluble in 0.1 M pyrophosphate at 30°, though not at about 25°, and they reconstituted only about 0.1–0.2% of the virus made by unacetylated protein at either temperature (Table III). The presence of more than one *O*-acetyl group also interfered with reconstitution. The failure of such preparations to reconstitute was shown on G-100 Sephadex (with 0.1 M pH 7.5 phosphate) to be correlated with a lower state of aggregation of these acetyl protein preparations compared to unmodified TMV protein (Colloms, 1966b).

Subsequent studies were performed with the protein isolated from acetylated virus, which was thus known to be acetylated predominantly at lysine 68, as well as on one tyrosine, presumably 139. (The *O*-acetyl group was found not to become released under the conditions of isolating the protein in 67% acetic acid.) This protein reconstituted about 74% of the expected amount of virus, in terms of pelletable infectious material after hydroxylamine treatment, and not much less without such treatment. Thus the acetyl groups on residues 68 and 139, which it was possible to introduce into the native virus, were shown not to interfere with the renaturation of the protein when passing from 67% acetic acid through the isoelectric state to solution at pH 7.5, nor with the aggregation of the renatured protein to virus rods. The fact that the final virus pellet had approximately the same specific radioactivity as the protein remaining in the supernatant (one-third of total) also showed that in such selectively mono-*N*- and -*O*-acetylated samples no selection of less acetylated material occurred during recon-

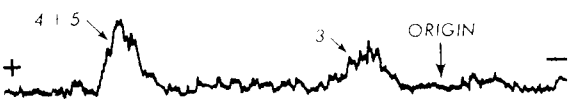

FIGURE 4: Autoradiographic scan of pH 9.2, 0.2 M sodium borate electropherogram of tryptic peptides obtained from acetyl-TMV. The scan shows that at this pH only two major radioactive peaks appear. Markers were used to identify the peptides. Apparently acetyl peptides 3 and 3a are not resolved in this system and they are both clearly separated from acetyl peptides 4 and 5.

TABLE III: Reconstitution of Acetylated Protein.[a]

Type of Protein	Acetyl/mole		Temp of Reconstitution (°C)	Infectivity (%)	Virus Pellet (%)
	N-Ac	*O*-Ac			
Acetylated protein	2.0	0.0	23	0.2	
	1.2	1.5	23	0.2	
	1.2	0.1	23	2.0	
	1.0	1.7	23	5.5	
	1.0	1.7	30	4.5	
	1.0	0.1	23	17	
	1.0	0.1	30	26	
	1.5	1.9	23	0.1	
	1.5	1.9	30	0.2	
	1.5	0.2	23	0.2	
	1.5	0.2	30	0.5	
	0.8	1.5	30	6	
	0.8	~0.1	30	40	54[b]
Protein from acetylated virus	0.6	1.0	30	47	
	0.6	0.0	30	100	74[b]

[a] Reconstitution was performed in 0.1 M (pH 7.2) pyrophosphate with about 1 mg/ml of protein and one-twentieth the amount of RNA for 6 hr at 30° or for 18 hr at 23 ± 2°. [b] The protein isolated from this reconstituted and twice-pelleted virus was digested with trypsin and the presence of acetylated peptides was determined. Peptide (4 + 5) contained at least 80% of the radioactivity, even though the unreconstituted acetylated protein preparation (54% pellet) contained similar amounts of acetyl peptides 3 and (4 + 5). This was not the case after acetylation of the virus (74% pellet).

stitution, nor was there any significant release of the more labile *O*-acetyl.

This was in contrast to the effect of reconstituting protein that had been randomly acetylated to the extent of about 0.8 *N*-acetyl. When the *O*-acetyl groups had been removed by hydroxylamine treatment, this protein reconstituted to virus with about 50% yield, and the protein in this virus proved, upon analysis, to contain almost only material acetylated at lysine 68, the 53 acetylated or doubly acetylated product having failed to become incorporated into the rod structure but being detectable in the nonpelletable protein (Table III).

Discussion

The first fact emerging from the present studies is that the presumed specificity of acetic anhydride from amino groups has little factual basis when considering ε-amino groups, since it seems that the pH range favoring acetylation of these and the phenolic groups is about the same (that of unprotonated amino and dissociated phenolic groups). That in actuality the reagent frequently achieves selective acetylation of the amino groups is probably due to the fact that these are usually more exposed than the phenolic groups of proteins. In the particular case of the TMV protein the fact that one tyrosine seems to react faster with acetic anhydride than the two lysines, suggests a relatively more available location for this tyrosine. Very similar conclusions have recently been reached by Smyth (1967) working on the acetylation of pituitary peptides.

Focusing our attention on the reactivity of the protein in the intact TMV particle we find that one tyrosine residue and lysine residue 68 react much more readily than other groups. Previous studies of the iodination of TMV have indicated that only one tyrosine, namely residue 139, reacted in intact TMV (Fraenkel-Conrat and Sherwood, 1967). This was attributed to the peripheral location of the C-terminal part of the peptide chain, as indicated by carboxypeptidase susceptibility, frequency of viable amino acid replacements (*e.g.*, serine at 138 is frequently replaced by phenylalanine), serological specificity of such mutants, etc. Although in the present study no attempts were made to locate the tyrosine acetylations because of the alkali lability of this bond, it appears justified to postulate by analogy that only tyrosine 139 would be readily acetylated in intact TMV.

The relative reactivity of lysine 68 is also in line with other data. Thus mutants with an amino acid replacement in residues 65 and 66 are serologically distinguishable from common TMV, while replacements at residues 59, 63, and 81 are ineffectual in this regard (Sengbusch, 1965; Regenmortel, 1967). It must also be noted that

various TMV strains were found to differ from one another in positions 64–67, but none in 68–79 (Wittmann-Liebold and Wittmann, 1965; Wittmann, 1965). Similarly, almost no amino acid replacements were detected in chemically evoked mutants in the neighborhood of the lysines except frequently at residues 63 and 65 (Funatsu and Fraenkel-Conrat, 1964; Wittmann-Liebold and Wittmann, 1963). It appears probable from these data that the folding of the peptide chain brings residues 64–67 comparatively near the surface and that this accounts for the relative reactivity of lysine 68. Though even this residue is far less reactive in the virus than in the isolated protein, it certainly is less masked than residue 53. It must also be recalled that in the virus the tyrosines in positions 70 and 72 do not react with iodine (Fraenkel-Conrat and Sherwood, 1967) which supports the concept that starting with residue 68 the chain enters into a tightly packed and probably important field of interactions.

In the isolated protein both lysines are similarly reactive, but are exceeded in reactivity by one tyrosine. Surely this is again residue 139 which upon iodination of the protein also reacts more rapidly than tyrosine residues 70 and 72 (Fraenkel-Conrat and Sherwood, 1967).

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