121. Tobacco Mosaic Virus as a Carrier for Small Molecules I. The Preparation and Characterization of a TMV/α-Melanotropin Conjugate[1])

by **Verena M. Kriwaczek, Alex N. Eberle, Martin Müller,** and **Robert Schwyzer**

Institut für Molekularbiologie und Biophysik der Eidgenössischen Technischen Hochschule Zürich, CH-8093 Zürich[2])
and Laboratorium für Elektronenmikroskopie I, Institut für Zellbiologie der Eidgenössischen Technischen Hochschule Zürich, CH-8092 Zürich

Dedicated to Prof. Dr. *P. G. Waser* on the occasion of his 60th anniversary

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Summary

The use of tobacco mosaic virus (TMV) covalently loaded with hormones or other small molecules for various purposes including receptor detection and isolation is proposed. The basic principle is that of *cooperative affinity interactions* involving (large) numbers of artificially introduced sites (*e.g.* hormones) of the modified virus on the one hand and membrane-bound sites (*e.g.* receptors) of a cell or of a cell-membrane particle on the other. In order to test the feasibility of such TMV/hormone conjugates, TMV carrying about 500 molecules of a biologically active α-melanotropin analogue was synthesized, and characterized by its aspect under the electron microscope, by its infectivity, its melanophore-stimulating activity, and its reaction with antisera against α-melanotropin. The observed hormonal activity is in accordance with the idea of cooperative affinity interactions.

The chemical nature of membrane-bound receptors for drugs and hormones is still an unsolved problem of molecular biology. This paper is the first of a series investigating the use of tobacco mosaic virus (TMV) covalently attached to hormones, drugs, and other comparatively small molecules for the study of receptors. First, some pertinent chemical properties of TMV and of polypeptide-hormone receptors are reviewed and the concept of cooperative affinity labelling is introduced. It is then demonstrated that some 500 molecules of an α-melanotropin (α-MSH) analogue can be covalently attached to the virus particle without destroying the essential chemical and biological properties characteristic of the virus and the hormone.

[1]) This work was supported by the *Swiss National Science Foundation.* Abbreviations: TMV = tobacco mosaic virus; EM. = electron microscopy; symbols for amino acids and peptides according to the IUPAC-IUB rules [1].

[2]) Address for correspondence.

Tobacco mosaic virus is an easily accessible, crystalline [2] nucleoprotein with the approximate composition $C_{1,717,845}$ $H_{2,703,504}$ $O_{568,711}$ $N_{481,912}$ $P_{6,390}$ $S_{2,130}$[3]). This natural product, molecular weight *ca.* $40 \cdot 10^6$ Daltons, is composed of a single-stranded ribonucleic acid genome with about 6,390 nucleotides and a capsid containing 2,130 protein capsomers. The capsomers are roughly cigar-shaped and one end of each is in contact with three nucleotides. This nucleoprotein is wound into a right-handed helix with 130 turns, a pitch of 2.3 nm, and a diameter of 4 nm (for the nucleic acid phosphodiester backbone); there are $16\frac{1}{3}$ capsomers per helix turn, pointing radially outwards. The resulting virus particle has the shape of a hollow rod with inner and outer diameters of 2 and 9 nm, respectively [3] *(Fig. 1 and 3)*. By adjusting the ionic strength and/or pH of the medium, the particle can be decomposed into protein and RNA and reassembled again; however, within certain limits of pH, ionic strength, and solvent composition, the virus particle is very stable [4].

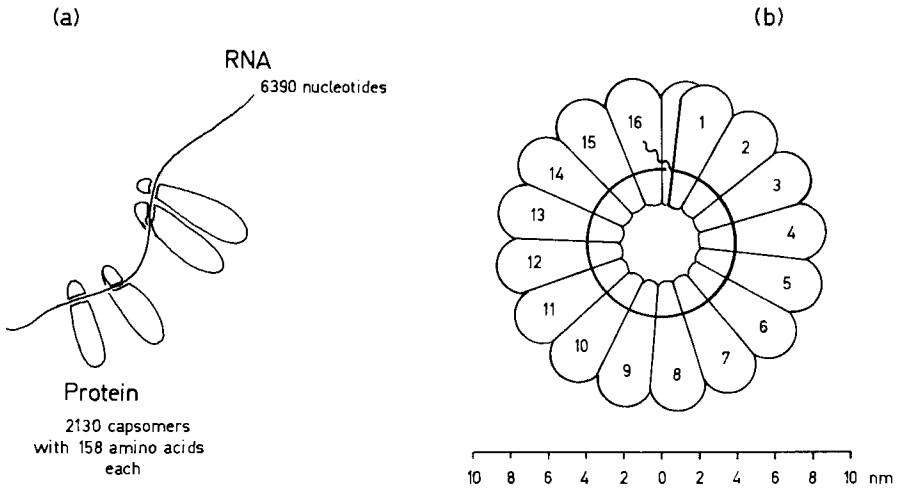

Fig. 1. *Organization of the RNA and the capsomers in TMV.* a) Hypothetical uncoiled nucleoprotein. b) An end-on view of TMV

Each wild-type capsomer consists of one $N(\alpha)$-acetylated polypeptide chain with 158 amino acid residues (C_{778} $H_{1,234}$ O_{245} N_{215} S_1, molecular weight 17,552 Daltons). The amino acid sequence (*vulgare* strain) is [5]:

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          10        20        30        40        50        60
          |         |         |         |         |         |
Ac·SYSITTPSQFVFLSSAWADPIELINLCTNALGNQFNTNNARTVVNRNFSNVWKPSPNVTV-

          70        80        90       100       110       120
          |         |         |         |         |         |
RFPDSDFKVYRYNAVLDPLVTALLGAFDTRNRIIEVENQANPTTAETLDATRRVDDATVA-

          130       140       150
          |         |         |
IRSAINNLIVELIRGTGSYNRSSFESSSGLVWTSGPAT.
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Of these amino acids, only the side-chain phenolic group of tyrosine Y139 and the amino group of lysine K68 are readily available for acylation in the intact virus, although an access by bulky reagents is hindered [6]. In the virus particle, the mercapto group of cysteine C27 is sterically hindered so that it is not modified by acylating agents. However, it is known to react with small molecules like methyl-

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[3]) Calculated on the basis of a ribonucleoprotein containing the 4 nucleotides in equal amounts and with protonated phosphate groups.

mercury nitrate to give an *S*-methyl-mercury derivative [7] and with iodine to give a stable (!) sulfenyl iodide [8] (iodine also reacts with tyrosine Y139, but not with Y2, Y70 and Y72 [9]).

Carboxy groups like those of aspartic acids D115 and D116 and of glutamic acids E131 and E145 are probably responsible for the binding of lead and calcium ions. An excellent review of TMV protein chemistry is provided by *Durham & Butler* who also propose a model for polypeptide chain folding in the capsomer [10].

Cooperative affinity labeling. If every capsomer of TMV carried a substituent group on its lysine K68 (which is located near the outer end of the capsomer [10]), the substituents would be about 2.3 nm apart in the axial and 3.5 nm in the circumferencial direction. In the case of large groups like the hormone α-melanotropin (α-MSH), these ligands would be rather crowded (the approximate length of an α-MSH molecule is between 2 and 5 nm, depending on the state of folding). In the examples discussed below, one TMV particle is loaded with 400–600 molecules of a α-MSH analogue. Every hormone substituent then has a mean TMV surface area of about 20–30 nm^2 at its disposal; it would be separated from its neighbors by roughly 5–7 nm. Motions due to chain flexibility should be practically unhindered by the other molecules of the α-MSH analogue.

Hormone receptors are believed to be integral membrane proteins with their hormone-receptive surfaces or subunits (the discriminators) exposed to the surrounding fluid of the hormone target cells. The cell surface may contain roughly 10^3–10^6 discriminators or up to an average number of 300–600 per μm^2 [11]. However, because of lateral diffusion in the plane of the plasma membrane, much higher local concentrations could be attained at certain times and under certain circumstances (*e.g.* in the case of the 'capping' phenomenon [12], where receptors become concentrated in one small area of the cell surface). TMV substituted with 500 hormone molecules has a surface concentration of about 30,000 substituents per μm^2. If one-third of the TMV surface came into contact with a receptor-bearing plasma membrane, about 160 hormone molecules spread over an area of 0.01 μm^2 would be available for contact with roughly 1–5 discriminators *(Fig. 2)*.

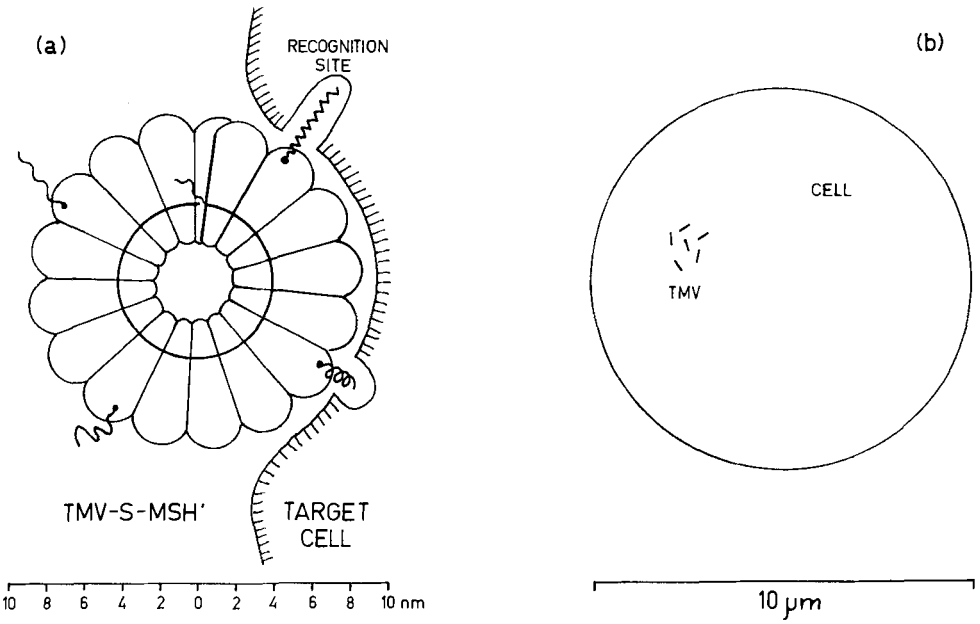


Fig. 2. *A possible interaction of hormone-substituted TMV with discriminator-bearing cells.* a) An end-on view of a hormone-substituted TMV particle (with polypeptides of the size of α-MSH attached to lysine K68 at a distance of *ca.* 7 nm from the virus axis) in contact with the cell surface. b) A comparison of the sizes of TMV and a spherical target cell with a diameter of 10 µm

If hormone-substituted TMV particles do react with hormone receptors (which is the case on α-MSH substitution [12]), then one particle could interact with several hormone receptors simultaneously (the number depending on the local discriminator concentration). Every discriminator would furthermore experience a 'local hormone concentration' of roughly 10^{-5}–10^{-6}M, which is high compared to the usual dissociation constants of hormone molecules (*ca.* 10^{-8}–10^{-10}M). Such a situation would be expected to favor binding and to enhance the overall association constant due to cooperative effects. TMV/hormone conjugates should therefore bind to discriminator- and receptor-containing membranes and membrane particles more strongly than individual hormone molecules. After disruption of the virus, the TMV-capsomer/hormone conjugates would be expected to display a lower binding force again. Evidence for enhanced association shall be produced below and in the following paper [14].

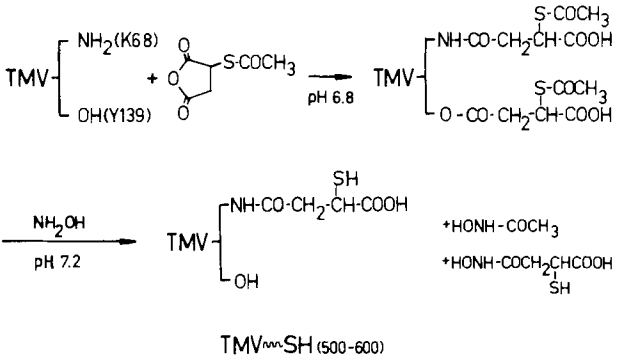
A very strong association would allow a *labeling* of receptor-bearing tissues, cells, and cell-membranes with substituted TMV.

Work on this aspect is under way: (i) Membrane vesicles with bound TMV/hormone conjugates can be demonstrated with the *electron microscope* [14]. (ii) The labeling produces *density changes* which can be used for the separation of discriminator-bearing membrane vesicles [14] (such an affinity density perturbation [15] can be enhanced if the virus is made to contain heavy atoms or groups like iodine or methyl-mercury in addition to the hormone substituents). (iii) In addition to hormone molecules a number of fluorescent groups (fluorescamine, 1-dimethylamino-naphthalene-5-sulfonyl chloride, fluoresceine isothiocyanate, and others) that allow the detection of discriminator-bearing cells under the *fluorescence microscope* can be introduced into the virus. (iv) TMV can be loaded with hormones and with carboranyl alanine [16] to convey *neutron-susceptible boron* atoms to discriminator-bearing cells.

*Preparation of (3-mercapto-1-succinyl)-TMV/[1-(bromoacetyl-*D*-alanine), 3-glycine, 4-norvaline]-α-melanotropin conjugate* (TMV〰S-MSH'). A unique point for a stable ligand attachment to TMV is certainly the ε-amino group of lysine K68. However, acylation by bulky ligands (and most biologically interesting molecules are rather bulky) appears to be precluded as pointed out above. We therefore modified lysine K68 with an intermediate link containing an easily accessible mercapto group. This group appeared to be most appropriate, because a number of very specific reagents are known to react with a mercapto group, and because the only intrinsic mercapto group of TVM, that of cysteine C27, is sterically very strongly hindered. Furthermore, such reagents containing bromoacetyl-, maleimido-, and mercapto-groups (reacting by way of nucleophilic substitution, addition, and disulfide-bond formation, respectively) can easily be introduced in peptide hormones and other ligand molecules.

In the examples given in this paper the mercapto-group was introduced *via* 2-acetylthio-succinic anhydride [17], although methyl 3-mercaptoproprionimidate

Scheme 1. *Preparation of (3-mercapto-1-succinyl)-TMV* (TMV〰SH)



probably would have done just as well [18]. The procedure is outlined in *Scheme 1.* TMV was reacted with a 25-fold excess of 2-([O = ^{14}C]-acetylthio)-succinic anhydride in phosphate buffer, of pH 6.8. Presumably, both the ε-amino group of lysine K68 and the phenolic hydroxyl of tyrosine Y139 reacted: at any rate, after dialysis about 800 [O = ^{14}C]-acetyl groups remained connected with the virus. Treatment with hydroxylamine at pH 7.2 completely removed the [O = ^{14}C]-acetyl groups and revealed at least 500–600 mercapto groups firmly attached to the virus as demonstrated spectrophotometrically [19]. As hydroxylamine attacks active esters very readily, we expect all substituents at Y139 to have been removed. Although the procedure introduces not only the mercapto group, but also changes the positive charged ammonium form of K68 into the negative charged carboxylate ion of the 3-carboxy-3-mercaptopropionyl group, the substituted virus particles remain intact *(Fig. 3).*

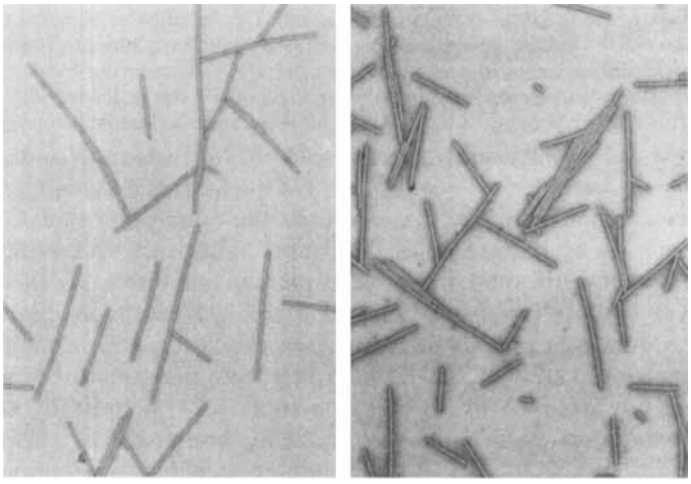
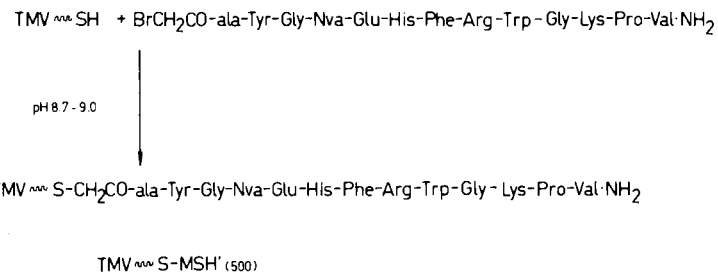


Fig. 3. *TMV (a) and (3-acetylthio-1-succinyl)-TMV (b).* An electron-microscopic view; negative stain with phosphotungstic acid, magnification 45,000×

Scheme 2. *Preparation of (3-mercapto-1-succinyl)-TMV/[1-(bromoacetyl-D-alanine), 3-glycine, 4-norvaline]-α-melanotropin conjugate* (TMV〰S-MSH')



In the example of this investigation the mercapto group was then reacted with [1-(bromoacetyl-D-alanine), 3-glycine, 4-norvaline]-α-MSH (= Br-MSH') [20] according to *Scheme 2.* After reaction and dialysis, a considerable amount of the

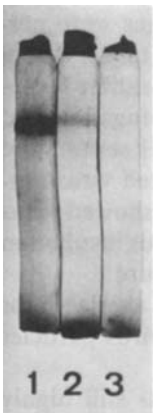


Fig. 4. *Gel electrophoresis of TMV〰S-MSH' at pH 4.5* (method [26]).
1) Br-MSH'. 2) Crude TMV〰S-MSH'. 3) TMV〰S-MSH' after purification by electrodialysis. TMV〰S-MSH' remains at the starting point (bottom) because of its size

α-MSH analogue remained non-covalently associated with the virus particle as was shown by gel electrophoresis *(Fig. 4)*. After removal of this component by electrodialysis in a β-alanine buffer of pH 4.5, approximately 500 ligand molecules remained firmly bound to the virus particle. This was demonstrated by using [1-(bromoacetyl-D-alanine), 2-(3′,5′-ditritio-tyrosine), 3-glycine, 4-norvaline]-α-MSH [20] as a label. With every attached ligand one additional negative charge (Glu) and two additional positive charges (Arg, Lys) are introduced into the virus at pH 7; yet the particle remains very stable *(Fig. 5)*.

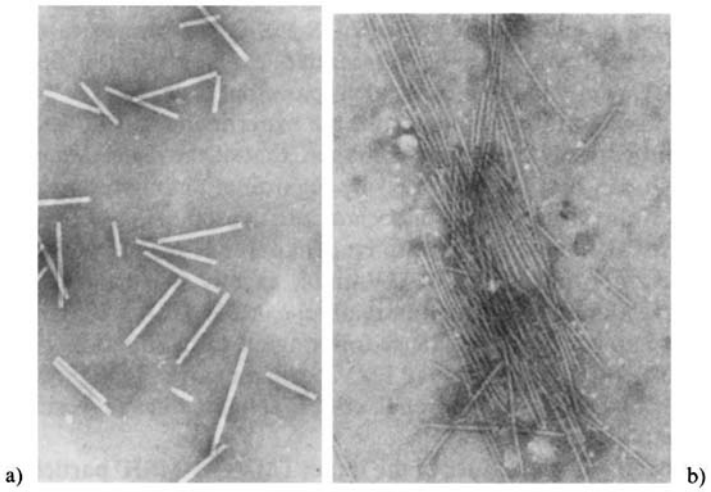


Fig. 5. *EM. views of TMV〰S-MSH'*. a) TMV〰S-MSH' + control serum (experiment no. 1), magnification 75,000×. b) TMV〰S-MSH' + antiserum 7 (experiment no. 11), magnification 45,000×

Reaction of TMV〰S-MSH' with antibodies against α-MSH. In order to test the accessibility of the ligand molecules on the virus particle surface, the reaction of TMV〰S-MSH' with antisera against α-MSH was investigated. The antisera had been prepared by injecting human serum albumin/α-MSH complex antigen into

rabbits and contained very high titers of specific antibodies [21]. Runs were performed with various combinations of TMV, TMV〰S-MSH', normal rabbit serum (control serum), two different rabbit anti-α-MSH sera, and goat anti-rabbit serum (see exper. part). After incubation, the TMV was separated by centrifugation and investigated with EM. Typically, the runs with TMV plus anti-α-MSH serum, and TMV〰S-MSH' plus normal rabbit serum showed only nicely separated virus particles; on the other hand TMV〰S-MSH' plus the anti-α-MSH sera showed virus aggregates with only very few single virus particles *(Fig. 5)*. Simultaneous incubation with goat anti-rabbit serum (='anti antiserum') did not change the picture.

The results indicate that the ligand on the TMV surface is readily available for reaction with even large molecules like antibodies; furthermore, all virus particles appear to be substituted with the α-MSH analogue.

Biological activities of TMV〰S-MSH'. The substituted virus was still highly infective for tobacco leaves (results of Dr. *Häni*, see exper. part) indicative of functional capsids.

In the isolated skin of *Rana pipiens*, TMV〰S-MSH' displayed a melanotropic activity of 10^7 U/g. This result demonstrates that the MSH' residues of the substituted TMV are accessible to the melanophore membrane discriminators. The time-course of the response generation was slightly slower than that of α-MSH. The maximum response level persisted for at least 3 h in contrast to that of α-MSH which diminished slowly after about 100 min. The disappearance of the response after removal of the hormone from the bath and during the wash-out phase was considerably retarded, and 20% remained even after 3 h (end of the experiment), whereas the α-MSH response reached zero level after 1 h. This result could indicate either physical trapping or endocytosis of TMV〰S-MSH' particles or capsomers. Strong cooperative affinity would be an alternative explanation for the retention of TMV〰S-MSH' activity in the biological preparation.

The extension and interpretation of these experiments is still in progress. Here, we wish to point out only one further problem, that of the *melanophoretic activity on a molar basis.* It can best be illustrated by regarding, among others, 3 different assumptions: a) If the virus were to dissociate into capsomers, and the 500 individual capsomer〰S-MSH' conjugates were to react like free H-MSH' molecules, then their hormonal activity would be $8 \cdot 10^8$ U/mmol, exactly 0.4 times that of H-MSH' (=[1-(acetyl-D-alanine),3-glycine,4-norvaline]-α-MSH [22]) which is a reasonable figure. We have no conclusive evidence for or against this situation. However, in the following report [14] it is shown that TMV particles carrying angiotensin II remain intact upon aggregation with adrenal cortex cell membrane vesicles containing angiotensin binding sites.

b) If about 160 MSH' residues of the intact TMV〰S-MSH' particle were to react each with an individual discriminator (receptor) as assumed in *Fig. 2*, the activity of every MSH' residue would be $2.5 \cdot 10^9$ U/mmol, about equal to that of H-MSH' [22]. However, this situation would require a confluent lateral diffusion or, alternatively, a 'sweeping up' by the virus of 160 discriminators into the membrane area covered by the TMV〰S-MSH' particle (*ca.* 0.01 μ^2) which, to this extent, appears to be rather improbable.

c) If less discriminators were present under the contact area of the virus, *e.g.* 1 to 3 (according to probability, see above), then the activity of the 1–3 individual, imme-

diately reacting virus-bound MSH' residues would be $4 \cdot 10^{11}$ to $1.3 \cdot 10^{11}$ U/mmol. This is between 200 and 70 times the activity of H-MSH' and 10 to 3 times that of α-MSH with the natural sequence.

Hence, in any of the situations encompassed by b) and c) in which less than 160 discriminators are activated by a single TMV〰S-MSH' particle, the reacting MSH' residues would be *super-active*. Such situations could be explained in terms of co-operative affinity and closely related concepts which are based on mechanisms enhancing the time-average probability of a discriminator being occupied by a hormone residue. However, detailed statements shall be postponed until we have more experimental information.

Experimental Part

Tobacco mosaic virus, TMV (wild type). Plants of *Nicotiana tabacum* were grown and infected by Dr. *Alfred Häni* of the Eidgenössische Forschungsanstalt für landwirtschaftlichen Pflanzenbau, Reckenholzstrasse 191/211, CH-8046 Zürich. The virus was extracted and purified according to *von Wechmar & van Regenmortel* [23]. A milky, strongly birefringent, very homogeneous suspension of long virus particles was obtained *(Fig. 3)*.

(3-Mercapto-1-succinyl)-TMV (TMV-SH). 2-Acetylthio-succinic anhydride was prepared according to *Klotz & Heiney* [17] in 88.9% yield, m.p. 75–76° (lit. 75–76°). 2-([O=^{14}C]-Acetylthio)-succinic anhydride was prepared by the same procedure, using [O=^{14}C]-acetyl chloride. Different crops of active anhydride (yields 72.8–74.9%) were mixed to produce a sample of $7.694 \cdot 10^6$ dpm/mmol.

A virus preparation containing 51.2 mg ($2.94 \cdot 10^{-6}$ mol) of TMV protein was suspended in 3 ml of a 0.125M phosphate buffer of pH 6.8, and slowly treated with a 25-fold excess of solid 2-([O=^{14}C]-acetylthio)succinic anhydride (524,450 dpm). After 8 h at 20° the mixture was cooled to 4° and dialysed 3 times for 6 h against phosphate buffer. The dialysate then contained 19.2 dpm/ml (*ca.* background value) and the virus compartment (5.6 ml) 1110 dpm/ml. Protein was assayed by the *Lowry* method [24]: 36.52 mg. The substitution density was 0.378 acetyl groups per capsomer or about 800 substituents in a complete TMV particle with 2130 capsomers. Application of a 50-fold excess of anhydride didn't change these figures appreciably.

The (3-acetylthio-1-succinyl)-TMV was treated for 45 min with 1.2 equiv. per substituent group of 0.01M hydroxylamine at pH 7.2 and 20°. The virus was dialysed against 0.125M phosphate buffer of pH 6.8 as above until only background amounts of radioactivity remained associated with it. EM. revealed undamaged virus particles. A spectroscopic assay of the number of exposed thiol groups with the DTBN reagent [5,5'-dithio-bis(2-nitrobenzoic acid)] [19] gave values of about 500–600 thiol groups per TMV particle. A certain variation is due to the inherent turbidity of the solutions.

(3-Mercapto-1-succinyl)-TMV/[1-(bromoacetyl-D-alanine),3-glycine,4-norvaline]-α-MSH conjugate (TMV〰S-MSH'). A mixture of 18 mg of $BrCH_2CO$ · ala-Tyr-Gly-Nva-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val · NH_2 (=3r-MSH') [20] and *ca.* 10 μCi of Tyr^2-tritiated Br-MSH' was dissolved in 100 μl of a 1% solution of acetic acid and treated brought to pH 8.7–9.0 with NaOH. It was then added to 1.7 ml of a TMV〰SH suspension in containing 10 mg of virus protein. After 4 h of slow rotation in a round-bottomed flask at 20°, the mixture was dialysed against 0.125M sodium phosphate buffer of pH 6.8 at 4° (3 times 3 l for about 4–6 h each). The virus particles remained intact during the procedure *(Fig. 5)*. They contained, in addition to covalently bound MSH', a physically adsorbed MSH'-like component that could not be removed by dialysis *(Fig. 4)*. Complete purification was achieved with electro-dialysis in β-alanine buffer at pH 4.5. The virus was placed inside a collodion bag containing the anode, and electro-dialysed for 1 h at 100 V and 10 mA in a large pot containing the cathode. The buffer was then removed by dialysis against water. No MSH' derivatives were detectable upon gel electrophoresis *(Fig. 4)*. After purification, the degree of substitution by MSH' was about 0.24 hormone molecules per capsomer or 500 per complete virus particle. The preparation showed MSH activity in the frog skin melanophore test [25]: about 10^7 U/g of virus particles.

Reaction of TMV〰S-MSH' with rabbit anti-α-MSH antisera. The rabbit anti-α-MSH antiserum 2 [21] as well as the antiserum 7, which was obtained in the same way as the antiserum 2 by immunizing rabbits with the complex 1 of [27], were both well-defined and very specific. The control sera were from non-immunized rabbits (gift of Dr. *H. G. Kopp*). 'Anti antiserum' was goat anti-rabbit serum (*Antibodies Inc.*, Davis, Cal., USA). The sera were incubated with the TMV preparations for 2 h at 38° and then for

1¾ h at 4°. The resulting mixtures were centrifuged for 10 min at 3000 rpm, the supernatant was decanted, and the pellets were dissolved in 10 µl of 0.125M phosphate buffer of pH 6.8. The sedimented material was viewed by EM. after negative staining with phosphotungstic acid.

The following runs were carried out with 50 µl of the specified sera and 0.08 mg (about 2 picomol) of the specified TMV preparation: 1) Control serum + TMV〰S-MSH'; 2) control serum + TMV〰S-MSH' + 5 µl anti antiserum; 3) control serum + TMV; 4) control serum + TMV + 5 µl anti serum; 5) antiserum 2 + TMV; 6) antiserum 2 + TMV + 5 µl anti antiserum; 7) antiserum 7 + TMV; 8) antiserum 7 + TMV + 5 µl anti antiserum. In all these runs the pellets consisted of pure, unaggregated TMV (or TMV〰S-MSH') rods *(Fig. 5)*.

The *crucial* experiments were the followings: 9) 50 µl Antiserum 2 + 0.08 mg TMV〰S-MSH'; 10) 50 µl antiserum 2 + 0.08 mg TMV〰S-MSH' + 5 µl anti antiserum; 11) 10 µl antiserum 7 + 0.087 mg TMV〰S-MSH'; 12) 10 µl antiserum 7 + 0.087 mg TMV〰S-MSH' + 5 µl anti antiserum. In all crucial experiments the pellets consisted of TMV〰S-MSH' aggregates *(Fig. 5)*. Very few unaggregated virus particles were to be seen. The addition of anti antiserum had no visible effect.

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