2. Melanotropin Receptors II. Synthesis and Biological Activity of α-Melanotropin/Tobacco Mosaic Virus Disulfide Conjugates[1])

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Dedicated to Prof. Dr. *Hans Neurath* on the occasion of his 75th birthday

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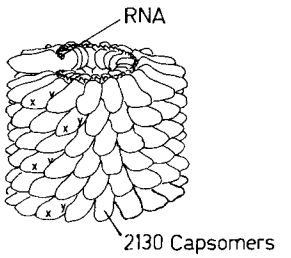
Asymmetric disulfide conjugates of mercaptosuccinyl tobacco mosaic virus (TMV ~ SH) with N^{α}-desacetyl-N^{α}-5-(mercaptovaleryl)-α-melanotropin were prepared *via* the *S*-sulfoderivative of the peptide. The conjugates, TMV ~ S–S ~ α-MSH(n), contained up to n = 330 disulfide-linked peptide molecules/virion. Similarly, fluorescent conjugates, Rh(m) ~ TMV ~ S–S ~ α-MSH(n) were prepared, containing $m \approx 200$ rhodamine molecules linked to the virions by thiourea bridges. Such conjugates were designed to study α-MSH receptor localization and dynamics (mainly internalization), because the carrier virions which served to enhance specific receptor binding and as fluorescent or radioactive markers may be detached from the neuropeptides at will by reduction. Reduction occurred in solution and on the cell surface, but not in the cytoplasm, thus allowing detection of internalized agonist-receptor complexes. The conjugates were superpotent agonists for tyrosinase stimulation in *Cloudman S-91* melanoma cell cultures, but were inactive for *cyclic* AMP accumulation. Their rather rapid internalization and the influence of reducing agents and other agonists on their biologic activity suggest a close connection between receptor location and biologic response as well as the presence of essential receptor HS-groups.

Introduction. – The recent discovery of regulatory peptides common to the nervous and endocrine systems has, because of its fundamental and practical implications [3], revived interest in peptides tremendously. Our laboratory is dealing with two aspects of neuropeptides and hormones: structure-activity relationships [4] and molecular mechanisms of peptide-receptor interactions, including the influence of lipid membranes [5]. This report is concerned with receptors, particularly with development of new tools for studying receptor localization and dynamics in tissue slices and target cells.

Virions of tobacco mosaic virus (TMV) carrying peptides covalently attached to a considerable number (50 to 500) of the identical capsomers *(Fig. 1)* react almost irreversibly with peptide receptors on a target cell surface to produce the biological effects typical of the attached peptides. Thus, they display properties expected of receptor-specific 'artificial antibodies' [6]. Such TMV-peptide conjugates, additionally labeled with fluorescent molecules, have been used for the study of cell-surface receptors with fluorescence microscopy [7]. In order to analyze the observed effects of receptor localization, clustering, patching, and internalization in more detail, it would be desirable to

[1]) Parts of this report have appeared as a thesis [1]. *P.M.* was on leave from the Chemistry Dept., Univ. of Athens; *S.D.S.* from the Central Drug Research Institute, Lucknow. Nomenclature and abbreviations, see [2] and *Experimental*. TMV is tobacco mosaic virus wild type.

$$\text{R-CH}_2\text{CO-Ser-Tyr-Ser-Met-}\overset{\ominus}{\text{Glu}}\text{-His-Phe-}\overset{\oplus}{\text{Arg}}\text{-Trp-Gly-}\overset{\oplus}{\text{Lys}}\text{-Pro-Val-NH}_2 \qquad \textbf{1–3a}$$
$$1 \quad 2 \quad 3 \quad 4 \quad 5 \quad 6 \quad 7 \quad 8 \quad 9 \quad 10 \quad 11 \quad 12 \quad 13$$



≡ TMV — x (y), x (y), x (y) **4**

Fig. 1. *Structural features of mammalian α-MSH, wild type TMV, and their derivatives.* **1**, R = H: α-MSH; **2**, R = $Br(CH_2)_3$: $N^{2,1}$-(5-bromovaleryl)-$N^{2,1}$-deacetyl-α-MSH; **2a**, R = $Br(CH_2)_3$, instead of Tyr(I) = 3-[^{125}I]iodotyrosine: $N^{2,1}$-(5-bromovaleryl)-$N^{2,1}$-deacetyl-$C^{3,2}$-[^{125}I]iodo-α-MSH; **3**, R = $^-O_3S–S–(CH_2)_3$: $N^{2,1}$-desacetyl-$N^{2,1}$-(5-(sulfothio)valeryl)-α-MSH; **3a**, [^{125}I]iodo-**3** (*cf.* **2a**). **4**, Subunit structure of TMV (only about 20% of the total virion length is shown); x and y indicate the relative positions of two of the functional groups chemically accessible on the virion surface (they occur on all capsomers): x is the N^{ε}-group of Lys-68 and y the HS-group of Cys-27. To indicate different types of virion substitution, we use the fork-type abbreviation (bottom, right) with –NH– or –S– in place of x or y, as necessary (see *Scheme 1*).

have TMV conjugates in which the carrier virion (fluorescent or radioactive) could be released from the peptide molecules at will and, hence, removed from the target cell surface after specific time intervals.

As a first example, we describe here the attachment of a biologically active α-melanotropin (α-MSH) derivative [2] by means of disulfide bonds. A quantitative release of the peptide from the virion was easily achieved by reducing agents, preferably dithiothreitol (= 1,4-mercapto-2,3-butanediol; DTT) or $NaBH_4$, under conditions that do not harm the living target cells.

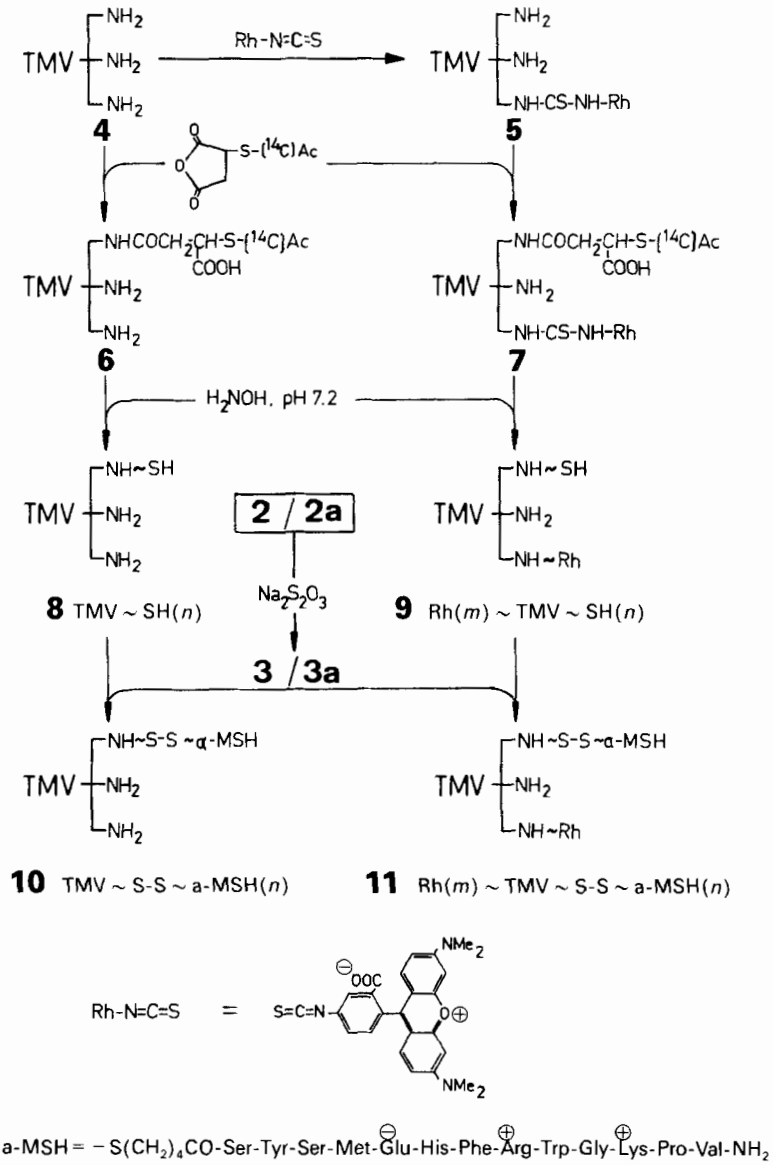
The use of these conjugates in α-MSH-receptor research and their expected and unexpected biological activities (see also [1]) shall be mentioned briefly; more detailed reports on the biological implications are to appear elsewhere.

Synthetic Results. – We prepared asymmetric TMV/α-MSH disulfide conjugates using *Swan*'s method for disulfide bond formation [8],

$$RSH + R'SSO_3^- \rightleftarrows RS–SR' + HSO_3^-,$$

as outlined in *Scheme 1*. The educts corresponding to RSH, mercaptosuccinyl-TMV (**8**) [10] and the strongly fluorescent rhodaminyl-mercaptosuccinyl-TMV (**9**; preliminary account in [7], details given here), were derived from TMV (**4**) isolated in gram quantitites according to [9]. These educts were made to contain between 300 and 400 easily accessible mercaptosuccinyl groups on the virion surface in addition to the 2130 naturally occurring, buried, and practically unreactive HS-groups of Cys-27 *(Fig. 1)*. The key peptide intermediate corresponding to $R'SSO_3^-$ was $N^{2,1}$-desacetyl-$N^{2,1}$-(5-(sulfothio)valeryl)-α-MSH (**3**) containing tracer amounts of its radioactive [^{125}I]iodo derivative **3a** [2] *(Fig. 1)*. This '*Bunte*-Salz' [11] **3**/**3a** was prepared from a mixture of 'cold'

Scheme 1. *Synthesis of TMV/α-MSH Disulfide Conjugates*



and radioactive $N^{2,1}$-(5-bromovaleryl)-$N^{2,1}$-deacetyl-α-MSH and $N^{2,1}$-(5-bromovaleryl)-$N^{2,1}$-deacetyl-$C^{3,2}$-[^{125}I]iodo-α-MSH, respectively (**2**/**2a**; preparation and biological activity [2]).

Condensations of **8** (or **9**) with **3**/**3a** were carried out in suitable buffers under N_2 using excess **3**/**3a**, similarly to the preparation of asymmetric disulfide conjugates of other proteins [12]. The sulfite scavenger $SrCl_2$ hardly increased the substitution rates *n*.

Phosphate and EDTA competed with SO_3^{2-} for Sr^{2+}; in *Tris*, precipitation of $SrSO_3$ became visible immediately after mixing the components **9** and **3/3a**.

Gel-permeation chromatography over *BioGel P-10* was found to be better suited for the purification of TMV derivatives **5–11** than precipitation, dialysis, and electrodialysis alone [10]. The pure conjugates appeared with the exclusion volume and were well separated from excess **3/3a** and other low molecular weight compounds *(Fig. 2)*. Disulfide-linked aggregates of the TMV derivatives **8–11** which may arise from inadvertent oxidation or disulfide exchange under alcaline conditions [8] [13] always remained at the very top of the columns as shown by electron microscopy and radioactivity measurements.

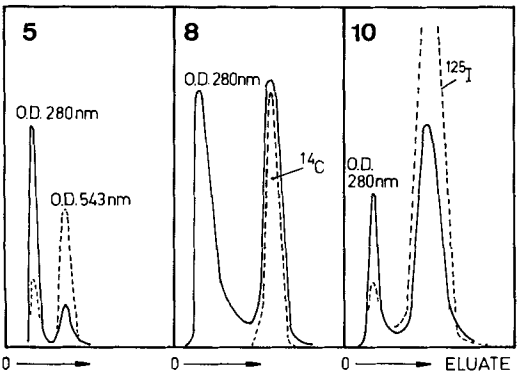


Fig. 2. *Purification of substituted TMV* **5**, **8**, *and* **10** *with gel-permeation chromatography. BioGel P-10* (200-400 mesh), 1 by 18 cm for 1.5 mg of virus-protein. Eluant: 125 mM phosphate, pH 6.5, fraction size 0.7 ml at 6.3 ml/h flow rate.

At present, our best method for preparing disulfide conjugates is to carry out the condensation in *Tris* buffer at 37° for 5 days, to apply the solution to a *BioGel P-10* column, and to elute with EDTA buffer at pH 6.5 in order to avoid disulfide exchange leading to symmetrical disulfides [8], including virion aggregates, and substituent losses. We are still studying further optimization of conjugate formation.

The conjugates were characterized by electron microscopy to ascertain their gross integrity and absence of bacterial contamination. As expected, they all proved to be indistinguishable from native TMV preparations and had the appearance of the examples reported in [10]. Our analytical basis was the protein content of the samples, determined according to *Lowry* [14]. Accessible HS-groups were estimated by *Ellman*'s method [15] and by reaction with tritiated *N*-ethylmaleimide. In **8** and **9**, the values were equal to or slightly lower than the number of *S*-[^{14}C]acetyl groups in **6** and **7** from which they were derived. With our methods, a maximum of about 400 mercaptosuccinyl groups per virion was regularly introduced.

The degree of substitution n of **10** and **11** with disulfide-linked peptides was estimated from radioactivity measurements (incorporation of ^{125}I). With mercaptosuccinylated virus containing between 300 and 400 accessible HS-groups, $n \approx 80$ to 100 was readily attained; in some favorable, but unexplained cases, n was as high as 400. Reproducibility in the high range was not easily controlled, because the excess of **3/3a** was not the only decisive factor. For our purposes, it was essential to be sure that the conjugates **10** and **11** did not contain non-covalently bound, adsorbed α-MSH (or

rhodamine) derivatives. This was conclusively demonstrated by denaturation of the pure virus preparations and gel electrophoresis [10].

The fluorescence marker rhodamine was introduced into TMV as one of its isomeric isothiocyanates. Thus, thiourea derivatives were obtained in which the rhodamine was most probably linked to the only readily accessible amino group of the capsomers, that of Lys-68. Quantification was not easy, but in all cases examined; the lysine content of **5** was lower than that of **4.** Semiquantitative estimation of the degree of substitution by rhodamine, *m*, in **9** was achieved by a difference method. We had observed that the maximum number of mercaptosuccinyl groups that can be introduced into a virion (about 400) is reduced by the number *m* of rhodamine groups present. Thus, **8** and **9** were prepared from **4** and **5** simultaneously under exactly the same conditions and the number of HS-groups determined by reaction with *N*-[^{3}H]ethylmaleimide and scintillation counting. The difference in counts was found to correlate well with the time of exposure of **4** to Rh–N=C=S *(Scheme 1)* and was therefore assumed to be a measure for the incorporation *m* of Rh–NH–CS groups.

As the reaction of isothiocyanates with an N^{ε}-amino group requires alcaline media and TMV shows reduced stability at pH > 8, we examined its behaviour in different buffers. Both TMV stability and reaction rate were better in 50 mM carbonate (pH 9.5, 37°) than in corresponding *Tris,* phosphate, and borate buffers. Using an eight-fold excess of Rh–N=C=S/capsomer, we found *m* to be about 100/virion after 15 min and about 200 after 30 min in a series of 4 experiments. Denaturation of **5** and gel electrophoresis showed a similar increase of fluorescence associated with capsomer protein (visual examination). Our preparations of **11** bound selectively to receptor-bearing target cells with enough fluorescence to make observations with the fluorescence microscope easy (see below and [7]). It even appeared *(Fig. 3)* that **11** crystallized in a manner similar to native TMV (*Stanley* [9]).

A few special features of **8, 9,** and **4** HS-group reactivity were revealed by control experiments *(Scheme 2)*. Whereas the 5-bromovaleryl group of **2**/**2a** did not react with **8** (*a*), the more reactive bromoacetyl group of [1-(bromoacetyl-D-alanine),3-glycine,4-norvaline]-α-MSH ($BrCH_2CO$-MSH') had been found to react very easily with **9** (→**12**;

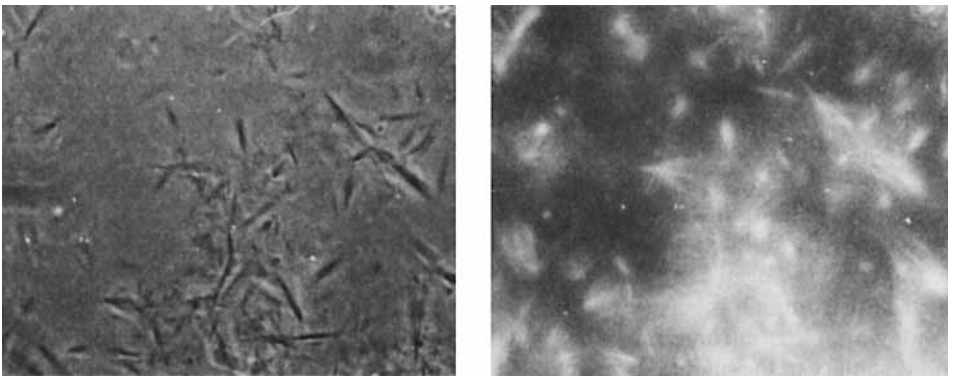
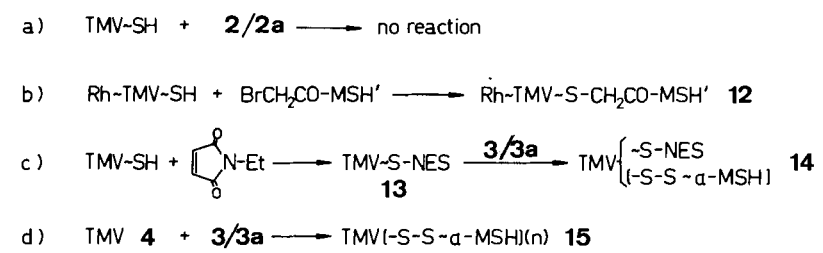


Fig. 3. *Rhodamine-TMV-peptide conjugate* **11** *in 800-fold magnification.* Phase contrast (left) and fluorescence microscopy (right).

Scheme 2. *Control Experiments of TMV Mercapto Group Reactivity*



b) [7] [10]. After blocking all the introduced mercaptosuccinyl groups of **8** with *N*-ethylmaleimide (→**13**), we had expected to find no reaction with **3/3a** (*c*); however, radioactivity was definitely introduced at a level of about 20 or less modified α-MSH molecules per virion. This conjecture was substantiated by the reaction of native TMV (**4**) with the '*Bunte* Salz'→**15**; (*d*). The rather inaccessible HS-group of Cys-27 [17], therefore, appeared to be quite inert towards *N*-ethylmaleimide, but to react with the (sulfothio)valeryl group. We cannot yet offer a satisfactory explanation for this difference, but may say that a disulfide bond appears to be formed between the α-MSH derivative and Cys-27, because reduction with $NaBH_4$ or DTT removed radioactivity from this product as well as from **10** *(Fig. 4)*. Such reductions were rapid with $NaBH_4$ and DTT being complete after 15 min at 37°, but slow with mercaptoethanol under the same conditions.

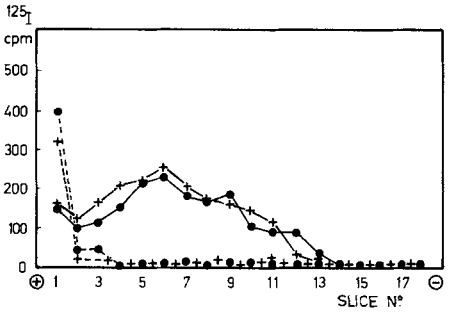


Fig. 4. *Reductive cleavage of* **10** (+++) *and* **15** (●●●). *SDS* polyacrylamide gel electrophoresis of **10** and **15**, applied as equal amounts of radioactivity, before (continuous lines) and after (broken lines) reduction with DTT (55 mM) for 15 min in phosphate buffer (pH 8.3) at r.t. After the reaction, TMV was isolated by precipitation with NaCl and poly(ethyleneglycol) and subjected to dissociating gel electrophoresis according to [16]. Radioactivity was determined in 0.5 cm slices of the gels.

Biological Results. – Four preparations of **10** with 55, 125, 188, and 330 peptide molecules per virion were investigated for tyrosinase stimulation, cell binding, and *cyclic* AMP accumulation in *Cloudman S-91* mouse melanoma cell cultures. Like our previous TMV sulfide conjugates with α-MSH and other peptides [6], these disulfide conjugates showed superpotency and superaffinity effects that increased with virion substitution. However, two important things became clear that had not been substantiated by our earlier work: the tyrosinase stimulation by **10** is completely dissociated from a (non-existent) stimulation of *cyclic* AMP accumulation, and the superaffinity effects appear to be caused by enhanced receptor-mediated virion internalization. These two effects, observed for the first time, are perhaps closely related.

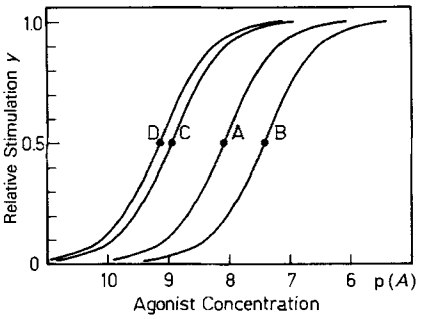


Fig. 5. *Log(dose)/response curves for tyrosinase stimulation in* Cloudman S-91 *melanoma cell cultures.* A: α-MSH, B: **2**, C: **10** (188), and D: **10** (330). Tyrosinase activity was determined after 48 h. For the conjugates, the agonist concentration shown (see p[*A*]) is that of the attached peptide molecules, not of virions. Calculations see [2], ED_{50} values and statistical data are in *Table 1.*

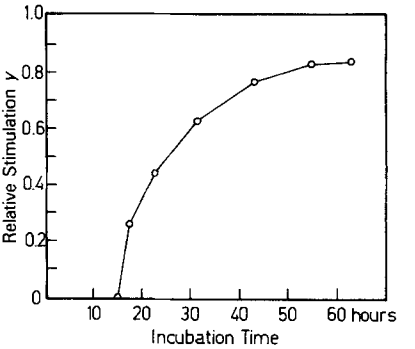


Fig. 6. *Time dependence of tyrosinase stimulation by* **10** (330) *in* Cloudman S-91 *melanoma cell cultures.* The plotted response is relative to that of α-MSH.

Superpotency for Tyrosinase Stimulation. The peptides attached to virions by disulfide links were roughly 10 times more potent than α-MSH or 100 times more potent than **2** from which they were derived *(Fig. 5)*. Tyrosinase stimulation is usually measured either 24 or 48 h after application of the agonist in order to bridge the sometimes considerable lag period ([2] and *Fig. 6*). In our cases, no significant differences were observed between the two measurements *(Table 1)*. We found the lag periods of our conjugates to be at least 3 times longer than those of α-MSH. Although we did not investigate this in detail, the potency of the conjugates appeared to increase with the number of peptide substituents, a general feature also of other TMV/neuropeptide conjugates (lit. in [6]).

Stimulation of Cyclic *AMP Accumulation.* The measurements were done as described [2] using 100 µl of extract as for the determination of background rates of *cyclic* AMP accumulation, *i.e.* 10 times more than for α-MSH or **2.** Even under such drastic

Table 1. *Agonist Potencies* (ED_{50}, nM) *for Tyrosinase Stimulation in* Cloudman S-91 *Melanoma Cell Cultures Determined after 24 and 48 h*

Agonist	Time	ED_{50}	S.E.M.	n[a])
α-MSH	24 h	5.37	1.30	7
	48 h	8.40	1.49	8
2	24 h	52.7	11.3	5
	48 h	40.7	0.55	6
10 (188)[b])	24 h	1.67[c])	–	1
	48 h	1.13[c])	0.045	2
10 (330)[b])	24 h	0.975[c])	0.045	2
	48 h	0.752[c])	0.002	2

[a]) Number of independent ED_{50} determinations with *Eqn. 1* in [2]. Each determination was based on 5 to 6 different agonist concentrations.

[b]) Number of α-MSH substituents per virion in parentheses.

[c]) Based on molarity of agonist (peptide) units, not virion molarity.

conditions that should have clearly revealed less than 1% of the stimulation caused by α-MSH, no effect was seen. We must conclude that our conjugates do not activate the adenylate cyclase receptor significantly.

Binding to Melanoma Cell Cultures. The time dependence of binding is shown in *Fig. 7* and *Table 2*. The conjugates **10**(55) and **10**(125) bound *ca.* 40 and 100 times, respectively, more slowly than α-MSH, but the number of peptide molecules becoming associated with the cells was 2 to 6 times higher. The dissociation of bound agonists

Table 2. *Agonist Binding to* Cloudman S-91 *Melanoma Cell Cultures*[a])

Agonist	B_{max} [cpm]	B_t [min^{-1}]	$t_{0.5}$ [min]	MSH/cell
α-MSH	506 (43)	0.39 (0.16)	1.8	3036
2	8302 (412)	0.25 (0.04)	2.8	49812
10 (55)	978 (108)	0.01 (0.003)	69.3	5868
10 (125)	3196 (104)	0.0036 (0.0002)	192.5	19176

[a]) Calculation according to *Eqn. 2* in [2]. $t_{0.5}$ is the time for half-maximal saturation, B_t the time constant, and B_{max} the maximal amount of radioactivity bound per cultured cell at saturation. S.E.M. in parentheses.

Table 3[a])

No.	Buffer	$SrCl_2$[b])	% Substitution	MSH groups/virion
1	Phosphate 125 mM	none	9.0	30.69[c])
2	Phosphate 125 mM	none	9.04	31.37
3	EDTA 10 mM	0.1	25.5	86.95
4	EDTA 10 mM	1.0	15.93	54.32
5	*Tris* · HCl 10 mM	1.0	25.06	85.45
6	*Tris* · HCl 10 mM	1.0	17.42	59.39

[a]) *Exper. 1–5* were at 37°/5 d, *Exper. 6* at 4°/14 h.
[b]) Equivalents with respect to $Na_2S_2O_3$ used.
[c]) This experiment was done with only 36.5 instead of 49 equiv. of **2** in the others.

from melanoma cells by incubation in agonist-free media is shown in *Fig. 8*. Initially, a certain amount of agonist was washed out very rapidly; we think that this portion was mechanically trapped in the cultures. Our assumption agrees with the fact that **10**(55) that had been allowed to bind for only 30 min, too short a time for appreciable association *(Fig. 7)*, was completely removed within 3 min *(Fig. 8)*. After the rapid initial stage, α-MSH dissociated more slowly, but completely, within *ca.* 30 min. Addition of DTT to cleave the disulfide bonds had a slight effect only in the first stages of conjugate dissociation, but did not reduce the 40% of radioactivity of **10**(125) remaining associated with the cells. We take this as a strong indication that the observed time dependence of fluorescence distribution [7] is indeed caused by receptor-mediated internalization. Inside the cells, the conjugates would be both inaccessible to DTT and unable to dissociate through the cell plasma membranes.

The fluorescent disulfide conjugates **11** behaved like the corresponding fluorescent thio-ether conjugates [7]. They labeled ~ 20–40% of the cells in a culture dish. If a 10-fold excess of non-fluorescent **10** was incubated simultaneously, fluorescent labeling

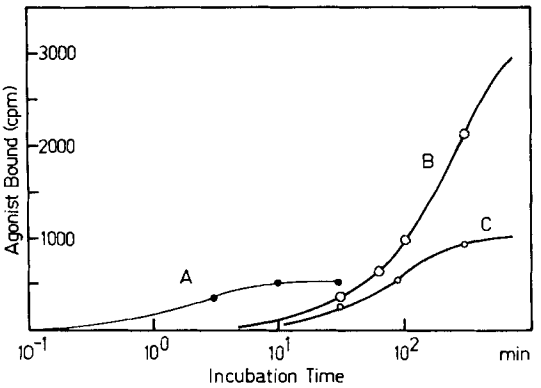


Fig. 7. *Time dependence of agonist binding to* Cloudman S-91 *melanoma cell cultures.* A: α-MSH, B: **10** (125), and C: **10** (55). Calculated parameters are in *Table 2.*

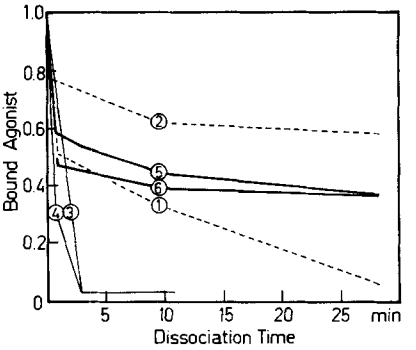


Fig. 8. *Dissociation of bound agonist from* Cloudman S-91 *melanoma cell cultures in BSS after 30 min agonist exposure* (see *Fig. 7*). ①: α-MSH; ②: **2**; ③: **10** (55); ④: **10** (55) in presence of 50 mM DTT; ⑤: **10** (125) after 1.5 h binding time; ⑥: as ⑤, but in the presence of 50 mM DTT.

was very strongly inhibited, showing a specificity for the same sites on the cells that apparently mediate binding and tyrosinase activity of **10.**

During the first 15 min of binding, the fluorescence on the cells had a patchy appearance and the reacting cells showed the typical surface ruffling observed earlier [7]. After about 15 min, the fluorescence became more and more diffuse with the cell nucleus showing quite clearly; in cells with correct orientation to the observer, capping phenomena were seen (*cf.* [7]).

If the cells were treated with DTT immediately after adsorption, most of the fluorescence disappeared and the cells remained only very weakly and diffusely labeled. A DTT treatment during adsorption, however, increased the labeling intensity and specificity (restricted number of cells with sharply defined patches) considerably, but did not impede the following stages of label capping and diffusion throughout the cells. Similar phenomena were caused by adding **2** (10^{-4}M) during the adsorption of **11.** This type of behaviour towards reducing agents and agonists was not observed with the thioether conjugates described earlier [7] [10]. We think it is a strong indication for the presence of essential HS-groups in the receptors.

Experimental Part

General. See [2]. Phosphate buffer was 125 mM (pH 6.5) unless specified. EDTA = ethylenediaminetetraacetate; *Tris* = tris(hydroxymethyl)aminomethane; DTT = dithiothreitol(= 1,4-mercapto-2,3-butanediol).

*(3-([^{14}C]Acetylthio)succinyl)-(Tobacco Mosaic Virus) (TMV ~ S[^{14}C]Ac(n); **6** (n)).* Products with *n* in the range of 200 to 400 (acetylthio)succinyl groups were prepared by a slight modification of the earlier procedure [10]. A soln. of TMV (wild type; 60 mg, 1.5 nmol of virions or 3.2 μmol of capsomers) in phosphate buffer (pH 6.8, 10 ml) was treated for 75 min at r.t. with small portions of a mixture of 3-(acetylthio)succinic anhydride (20 mg) and 3-([^{14}C]acetylthio)succinic anhydride (7.05 mg containing 0.8 μCi or 1.8×10^6 dpm ^{14}C). Thus,

the molar ratio of total reagent (156 μmol) to capsomers was 49:1. After 20 h, the mixture was dialyzed twice against phosphate buffer (3 l each time) and the TMV derivative precipitated with poly(ethyleneglycol) (*Carbowax, M_r* 6000) and NaCl (4 g/100 ml each). Final purification was achieved by gel-permeation chromatography through *BioGel P-10 (Fig. 2)*. The column size (1 by 18 cm) is adequate for the purification of up to 2 mg of virus protein (estimation according to *Lowry* [14]). The column may be stored after washing with buffer containing 0.1% NaN_3 to prevent bacterial growth. Before use, the azide must be eluted with fresh buffer (control by UV absorption) to prevent interference with biological assays. In this particular experiment, the radioactivity of the isolated product **6** indicated an incorporation of 346 3-(acetylthio)succinyl groups per virion (n = 346). The degree of incorporation depends to some extent on the excess of anhydride [10].

*Mercaptosuccinyl-(Tobacco Mosaic Virus) (TMV ~ SH(n); **8**(n))*. A soln. of **6** (8.8 mg, 0.22 nmol of virions, 76 nmol of (acetylthio)succinyl groups) in phosphate buffer (pH 6.8, 2 ml) was purged with N_2, adjusted to pH 7.2 with NaOH (1N, 56 μl), and treated with $NH_2OH \cdot HCl$ (0.1M, 76 μl, 100 equiv. per (acetylthio)succinyl group). After 45 min at r.t., the substituted virus was precipitated by adding phosphate buffer containing 8% (weight: volume) each of NaCl and poly(ethyleneglycol) (2.1 ml), collected by centrifugation, and resuspended in phosphate buffer (1 ml). This soln. was chromatographed through *BioGel P-10 (Fig. 2)*. TMV ~ SH(n) appeared with the exclusion volume; it was isolated by precipitation and centrifugation and resuspended in phosphate buffer (pH 6.5). Estimation of SH-groups with *Ellman*'s reagent [15] (the native SH groups of Cys-27 *(Fig. 1)* do not react) and of protein with *Lowry* reagent [14] indicated the virus preparation to be TMV ~ SH(327) with a recovery of 85% based on protein.

*TMV ~ S–S–$(CH_2)_4$CO-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2(n) (TMV ~ S–S ~ α-MSH(n): **10**(n))*. A soln. of **2** (1 mg, 0.538 μmol, 48.9 equiv. with respect to TMV-bound SH-groups) and **2a** (34 μl of a soln. obtained as in [2], neutralized with 5.9 μl 1N NaOH, about 1.8×10^7 cpm) in 14.5 μl of DMF was treated with $Na_2S_2O_3$ (51 μl, 0.1M; pH 8.1) and left to stand at r.t. for *ca.* 100 min. The mixture was then purged with N_2 and mixed with a suspension of **8**(341) (1.29 mg, 3.23×10^{-11} mol of virions, 1.1×10^{-8} mol of HS-groups) in 300 μl of the appropriate buffer (see *Table 3;* pH 7.2), purged thoroughly with N_2 (excess thiosulfate proved to be harmless in the condensation step and was not removed). The mixture was kept at the appropriate temp. (37° or 4°, see below) for up to 5 days and gently agitated once or twice daily to keep TMV in suspension. Radioactivity was monitored after each following step as required. The conjugate was precipitated by addition of a 16% poly(ethyleneglycol) and 16% NaCl soln. to a final concentration of 4% each and obtained as a pellet by centrifugation at 10000 rpm for 10 min. The pellet was resuspended in 200 μl of the appropriate buffer and applied to a column (1.3 by 17 cm) of *BioGel P-10* equilibrated with the same buffer. Fractions of 1 ml were collected at a flow rate of 12 ml/h. The conjugate appeared in the exclusion volume between 6 and 9 ml of eluate (UV monitoring at 280 nm). The fractions were pooled and the conjugate precipitated and gathered as above. The pellet was resuspended in 300 μl of the appropriate buffer and the radioactivity and the protein content *(Lowry)* measured to calculate the degree of substitution. Results in *Table 3*.

*Rhodamine(m) ~ TMV ~ S–S ~ α–MSH(n) Conjugate (**11**(m,n); cf. [7])*. A suspension of **4** (25 mg, 1.3 μmol capsomers) in phosphate buffer (pH 6.8, 0.5 ml) was added to Na_2CO_3 buffer (50 mM, pH 9.5, 4.5 ml); the resulting mixture had pH 9.25. It was treated at 37° with a solution of rhodamine isothiocyanate (isomer R, *Becton Dickinson AG*, Basel; 4.55 mg, 10 μmol, 8 equiv./capsomer) in DMSO (0.1 ml). After 30 min, the reaction was stopped by adding NH_4Cl (1M, 0.125 ml, 12-fold excess over rhodamine isothiocyanate) and the virions isolated in the usual manner by precipitation and centrifugation. The pellet was resuspended in 5 ml of phosphate buffer and dialyzed in the dark against 2 l of the same buffer. After 15 h, this crude preparation of **5** was used for reaction with 3-(acetylthio)succinic anhydride as described for **6**. The next steps leading to **9** and **11** were carried out as described for **8** and **10.**

The numbers of rhodamine (m) and HS-groups (n) of **9** were estimated with the following difference procedure: **8** and **9** were prepared simultaneously under exactly the same conditions. Aliquots of the two preparations (1 mg) were suspended separately in phosphate buffer (pH 6.0; 1 ml) and treated for 1.5 h at r.t. with a two-fold excess of *N*-[^{3}H]ethylmaleimide *(New England Nuclear Corp.)* with respect to acetylthio groups of **6** and **7**. The products were purified by precipitation *etc.* as described for **10** and their radioactivity determined by scintillation counting. For this and similar preparations, the difference indicated the presence of $m \approx 200$ Rh–NH–CS groups per virion.

Gel Electrophoresis was performed according to *Kato et al.* [16] with a 12.5% polyacrylamide gel containing 0.1% SDS and 8M urea. The conjugates (50–100 μg of protein in 50 μl of appropriate buffer) were incubated for 30 min at 50° with 24 mg of urea, 5 mg of SDS, and bromophenolblue (5 μl, 0.02%) prior to application.

Amino-Acid Analysis. Because of RNA acid stability, TMV products were hydrolyzed with concentrated $Ba(OH)_2$ for 24 h at 115° in a degassed and sealed ampoule. After cooling, the contents were acidified to pH 6

with dil. H_2SO_4. The precipitate was removed by centrifugation and washed, the supernatant was lyophilized, and the dry residue dissolved in the appropriate buffer for amino-acid analysis according to *Stein* and *Moore* (courtesy of Prof. Dr. *H. Zuber*).

Electron Microscopy of TMV and its derivatives was performed with negative contrast. A drop of virus suspension was applied to a carbon-copper grid, treated with phosphotungstate soln. at pH 7, and air dried.

Biological Assays. Cultures of *Cloudman S-91* murine melanoma cells and assays for binding, tyrosinase stimulation, and *cyclic* AMP accumulation are described in the foregoing report [2]. For the binding assays (*Fig. 7* and *Table 2*), radioactivity and peptide concentrations were: 5×10^5 cpm/ml for α-MSH (5×10^{-9}M), **10**(55) (1×10^{-10}M), and **10**(125) (4×10^{-11}M); 2.5×10^5 cpm/ml of **2**/**2a** were used (5×10^{-9}M).

Fluorescence labeling of Melanoma Cell Cultures with **11.** For fluorescence microscopy, the cells were grown on cover slips of glass or polystyrene that had been treated with polylysine (0.1 mg/ml) to facilitate cell adhesion [18]. For light-transmission microscopy, slips of other translucent plastics will do (*e.g. 'Thermanox'*). Two coated cover slips were transferred to a 6-cm *Petri* dish. Six dishes were seeded with the trypsinized cells of a 75-cm^2 culture flask in 30 ml of complete medium and incubated to coalescence. The cell cultures were washed twice with cold BSS and incubated with fresh BSS at 0° for 10 min. After removal of the BSS, the cells on the slips were covered with 50 µl of a soln. of **11** (or of control conjugates) containing 20–80 µg of TMV protein per ml of BSS. The covered dishes were kept at 0° for 60 min to allow adsorption. The cultures were then washed with cold BSS and incubated for different lengths of time with fresh BSS at 37°. After a wash with cold BSS, they were fixed with glutaraldehyde (2.5% in BSS) and embedded in glycerol/BSS 9:1.

Treatment with DTT was carried out either during the adsorption phase (0°, 60 min) or after. During adsorption, DTT in BSS (100 mM, 50 µl) was added along with the solution of **11** and the procedure completed as above. For investigating the effects after adsorption, the cells were washed briefly with BSS and then incubated for 20 min at 0° with a solution of DTT in BSS (100 mM, 50 µl), washed again with cold BSS, and treated and fixed as above.

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