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# BIOCHEMICAL ASPECTS OF THE VISUAL PROCESS

## XXII. AMINO GROUP MODIFICATION IN BOVINE ROD PHOTORECEPTOR MEMBRANES*

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SUMMARY

1. The role of primary amino groups in the structure and function of cattle rod photoreceptor membrane was investigated by determining the effect of chemical modification of these groups on the spectral properties and recombination capacity of rhodopsin and on retinol dehydrogenase activity.

2. Modification was achieved in two ways: (a) through amidination by reaction with methyl acetimidate at pH 7.5; (b) through trinitrophenylation with 1,3,5-trinitrobenzene-2-sulfonate (TNBS) at pH 8.0.

3. Amidination in darkness of up to 80% of the primary amino groups has little effect on spectral integrity and recombination capacity of rhodopsin and on retinol dehydrogenase activity. Amidination up to 98% leaves 70% of the rhodopsin and its recombination capacity intact, while retinol dehydrogenase activity is abolished.

4. Amidination in light causes a slow but ultimately complete loss of recombination capacity and a variable loss of retinol dehydrogenase capacity. After 98% amidination fewer lysine and more phosphatidylserine residues remain unmodified than after amidination in darkness.

5. Trinitrophenylation at 40 °C leads, both in darkness and in light, to complete modification with gradual loss of spectral integrity (in darkness), of the recombination capacity of rhodopsin and of retinol dehydrogenase activity.

6. These findings lead to the following conclusions: (a) Introduction of the small positively charged acetimidine group has little effect on the structure of rhodopsin, in sharp contrast to the introduction of the large uncharged trinitrophenyl group. (b) After illumination the chromophore-carrying lysine residue presumably returns to its original position. (c) Retinol dehydrogenase present in the photoreceptor membrane contains a catalytic amino group, which could bind the substrate *via* an aldimine bond. (d) Some of the phosphatidylserine residues of the membrane appear to be closely associated with rhodopsin.

Abbreviation: TNBS, 1,3,5-trinitrobenzene-2-sulfonic acid.

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## INTRODUCTION

The membrane of the vertebrate rod sacs, the photoreceptor membrane, is physiologically and morphologically rather unique. It contains the photopigment, rhodopsin, which upon illumination appears to increase the ionic permeability of the membrane[1]. Rhodopsin accounts for about 85% of the membrane protein[2–4]. The lipid phase is highly fluid, due to a low cholesterol content and an exceptionally high amount of polyunsaturated fatty acids in the phospholipids[5–8], and this permits the rhodopsin molecule a high degree of rotational freedom[9,10].

For a fuller understanding of the structure and functioning of the photoreceptor membrane a knowledge of the primary amino groups seems to be important. There are at least four ways in which these groups could participate. First, the characteristic absorbance spectrum of rhodopsin is due to the chromophoric group, 11-*cis*-retinaldehyde[11,12] linked by means of a protonated aldimine bond[13–16] to the ε-amino group of a lysine residue of opsin[17–19]. Secondly, the ease with which in synthetic retinylidene aldimines retinaldehyde transiminizes from one amino group to another[17,20] suggests that after illumination the isomerized chromophoric group may move by specific transiminization to other amino groups. Thirdly, primary amino groups could play an important role in enzymatic activities present in the photoreceptor membrane, like retinol dehydrogenase[21,22]. Fourthly, amino groups contribute to structure and stability of the membrane through their role in electrostatic interactions.

Selective chemical modification of amino acid side chains in protein is a valuable tool in obtaining information on protein structure and on "active center amino acids"[23,24]. Similarly, modification of free amino groups could yield information on their role in the structure and function of the photoreceptor membrane. Hence, we investigated the influence of chemical modification of the amino groups on photoreceptor membrane properties. This report deals with the effect on some properties of rhodopsin and of retinol dehydrogenase.

## MATERIALS AND METHODS

### *Materials*

1,3,5-Trinitrobenzene-2-sulfonic acid (TNBS) tetrahydrate was obtained from B.D.H., Ltd, Poole, Great Britain, as were 1-dimethylaminonaphthalene-5-sulfonyl chloride (dansyl chloride), dansyl amide, ε-*N*-dansyllysine, *N*-dansylserine, dansylglycine, α-*N*,ε-*N*-didansyllysine and *N*,*O*-didansyltyrosine.

*O*-Dansyltyrosine was prepared from *N*-carbobenzoxytyrosine benzyl ester (kindly supplied by Drs J. Th. W. A. R. M. Buis, Department of Organic Chemistry, University of Nijmegen) by reaction with dansyl chloride in acetone–0.2 M bicarbonate (4:1, v/v) at 37 °C. After evaporation, the protective groups were removed in 6 M HCl. The resulting *O*-dansyltyrosine was not further purified.

*N*-Dansylethanolamine was synthesized from equimolar amounts of ethanolamine and dansyl chloride in acetone–0.2 M bicarbonate (1:1, v/v) at room temperature. After completion of the reaction, the acetone was removed by evaporation and the water layer extracted with chloroform. The chloroform solution was washed (bicarbonate, dil. HCl, water to neutrality) and evaporated. The residue consisted

of *N*-dansylethanolamine and a small contamination of dansyl amide, which was removed on a silica gel column (Bio-Rad, *minus* 200 mesh) with ethyl acetate–cyclohexane (1:2, v/v) as eluent, which elutes dansyl amide first. The eluates, containing chromatographically pure *N*-dansylethanolamine, were combined, evaporated and the residue crystallised from a small amount of benzene, yielding pale-yellow crystals, melting at 101–102 °C, a value also reported elsewhere[25].

Methyl acetimidate hydrochloride was prepared according to Hunter and Ludwig[26]. The product was stored in dry air over KOH at 4 °C.

*Isolation and characterization of rod outer segment membranes*

Bovine rod outer segments are isolated as previously described[27]. Electron microscopy shows them to be very pure, and enzymatic studies confirm the absence of mitochondrial contamination, since no succinate dehydrogenase could be detected.

The rhodopsin content of normal and modified preparations is expressed as $\Delta A^1{}_{500}$, *i.e.* the decrease in the absorbance at 500 nm for 1 mg lyophilisate dissolved in 1 ml detergent solution upon illumination in the presence of 50 mM hydroxylamine, determined as previously described[5].

The recombination* capacity of the preparations is defined as the ratio of the $\Delta A_{500}$ value of the sample after illumination and incubation with excess 11-*cis*-retinaldehyde to the $\Delta A_{500}$ value of the original sample. Experimental details are given elsewhere[27].

Retinaldehyde is determined by the thiobarbituric acid method[28], modified according to Daemen *et al.*[2]. All-*trans*-retinaldehyde serves as a standard.

Retinol dehydrogenase is assayed with endogenous retinaldehyde serving as the substrate at 37 °C in 0.067 M phosphate buffer, pH 6.5. After illumination of the membrane suspension to "liberate" the retinaldehyde, a 5-fold molar excess of NADPH is added at $t=0$ and the amount of retinaldehyde remaining after various time intervals is determined as described above. Initial velocities were calculated.

Protein contents are calculated from amino acid analyses, performed as previously described[2].

Phosphorus is determined by means of a modified Fiske–SubbaRow method[6].

Gel electrophoresis is performed in sodium dodecyl sulfate on polyacrylamide as previously described[2].

*Trinitrophenylation*

(*A*) *Determination of primary amino groups.* Primary amino groups are determined spectrophotometrically with TNBS by a modification of the method of Habeeb[29] by performing the reaction in 1% Triton X-100–0.2 M borate buffer (pH 8.5). For the $\varepsilon_{340}$ a value of $13\,100 \pm 400$ is used (see Results).

(*B*) *Modification of primary amino groups.* Trinitrophenylation of photoreceptor membranes is carried out in suspension in 0.2 M bicarbonate (pH 8.0). The extent of modification is calculated from the 340-nm absorbance.

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* In a previous article[27] we called this property: regeneration capacity. Since the reaction involves only the last step of the *in vivo* regeneration process, we think that it is better described by the term: recombination capacity.

*Amidination*

Amidination is performed in aqueous membrane suspension at room temperature. Lyophilized membranes (10 mg/ml) are suspended in 0.2 M phosphate buffer (pH 7.5) by homogenization in a Potter–Elvehjem tube. An equal amount of methyl acetimidate hydrochloride (20-fold molar excess with respect to amino groups) is added and the pH is brought to 7.5 again by cautious addition of 1 M NaOH under stirring. After 0.5 h most of the reagent has been consumed by reaction with amino groups or by hydrolysis.

The soluble reaction products, mainly acetamide and methanol are then removed by sedimentation of the membrane fragments (80000 × *g*, 4 °C, 30 min). The sediment is either resuspended in the phosphate buffer to start a new cycle, or it is washed twice with double-distilled water and stored, with or without prior lyophilization, at −70 °C in a light-tight container. In order to achieve more than 95% modification, the amount of methyl acetimidate added during the fourth and fifth cycle should be doubled and quadrupled, respectively. Membrane fragments, which have been stored frozen, give similar results upon amidination as lyophilized preparations.

*Dansylation*

Dansylation is performed according to Gray[30]. To a suspension (5–10 mg/ml) of normal or modified rod outer segment membranes in 0.2 M $NaHCO_3$ (pH 8.0) an equal volume of a solution of 3–4 mg/ml dansyl chloride in acetone is added. After thorough mixing, the resulting suspension is shielded from the light and shaken for 0.5 h at 37 °C. In order to hydrolyse excess dansyl chloride the suspension is kept for some hours at room temperature. Then 5–10 vol. of 0.2 M $NaHCO_3$ are added in order to retain the lipids in the membrane fraction during the subsequent centrifugation (130000 × *g*, 4 °C, 30 min). The sediment is washed twice with bicarbonate solution, twice with double-distilled water, and is lyophilized and stored at 4 °C.

*Analysis of dansyl derivatives*

For subsequent analysis 0.5 mg dansylated membrane preparation is hydrolysed *in vacuo* in 6.7 M HCl for 18 h at 110 °C[31]. HCl is removed by evaporation *in vacuo* and the sediment is taken up in methanol–conc. $NH_3$ (95:5, v/v). Unmodified material is taken up in 1 ml, modified membranes in 150 µl. The solutions are stored at 4 °C.

Thin-layer chromatography is performed on silica gel G (Merck, 0.25 mm) with the solvent system chloroform–methanol–acetic acid (15:4:1, by vol.)[31] (Solvent system I). Of all systems tried[31,32], this is the only one which gives a good separation of ε-*N*-dansyllysine ($R_F$ 0.13), *O*-dansyltyrosine ($R_F$ 0.22), dansylic acid ($R_F$ 0.26) *N*-dansylserine ($R_F$ 0.32) and *N*-dansylethanolamine ($R_F$ 0.80). For quantitative determination of *N*-dansylethanolamine, however, this system is less suitable, since this compound can barely be distinguished from dansyl amide (a hydrolysis artefact with $R_F$ 0.82). For this purpose Solvent system II is used: ethyl acetate–cyclohexane (3:2, v/v)[31], in which dansyl amino acids do not move and *N*-dansylethanolamine ($R_F$ 0.15) is clearly separated from dansylamide ($R_F$ 0.35).

Before development the plates are equilibrated for at least 15 min in the saturated chamber. After chromatography they are dried in the air and sprayed

thoroughly with a mixture of triethanolamine and isopropanol (1:4, v/v)[31]. The spots are localized under near-ultraviolet light (350 nm).

For quantitative analysis, increasing volumes (2, 4, 6 and 8 $\mu$l) of each sample are applied to two plates, together with equal amounts (*i.e.* 0.25–1 nmole) of the reference substances, $\varepsilon$-*N*-dansyllysine, *N*-dansylserine and *N*-dansylethanolamine, (0.05 mg/ml in methanol–conc. $NH_3$). After development in either System I or System II the spots are scraped off, an equal area being taken for each set of spots of the same dansyl compound. The material is extracted overnight in the dark with methanol–conc. $NH_3$ (4–10 ml, depending on the fluorescence intensity).

Subsequently, the silica gel is sedimented (3000 × *g*, 10 min) and the fluorescence of the supernatants is measured relative to the references on a Beckmann Ratio Fluorimeter (excitation wavelength 360 nm, a Scott-Jena G3 filter, cutting off all wavelengths under 410 nm, is placed in the emission beam). The four spots for a given compound normally yield straight lines, from the slopes of these lines and the slopes of the lines of the reference substances, the amount of dansyl compound present can be calculated.

## RESULTS

### *Choice of modifying reagents*

For a proper evaluation of the effects of chemical modification of specific protein side chains, side effects like alterations of protein conformation should be prevented as much as possible. In order to investigate the role of primary amino groups in the photoreceptor membrane, we therefore looked for a mild amino group reagent. Methyl acetimidate, introduced by Hunter and Ludwig[26], seemed to suit our purposes almost perfectly. It reacts already under mild conditions (pH 7–10), exclusively with primary amino groups[26], and forms an acetamidine group, which is not much larger than the original amino group and which has at physiological pH the same charge as this group (Fig. 1a). Hence, even extensive amidination affects protein conformation only to a small extent[24].

Disadvantages of methyl acetimidate are its relatively rapid hydrolysis by water and the instability of the resulting amidine group towards acid or alkaline hydrolysis. The first problem can be overcome using successive amidination steps. The second problem means that the extent of modification cannot be determined directly by amino acid analysis, but must be calculated from the number of remaining amino groups.

The number of remaining amino groups cannot be determined by ninhydrin since this reagent appears to react also with amidine groups. Sörensen titration does not give reproducible results with small numbers of amino groups (less than 2 $\mu$moles) due to slow oxidation of the formaldehyde reagent to formic acid. We, therefore, investigated the suitability of TNBS for the determination of primary amino groups (Fig. 1b). This reagent has previously been applied for this purpose[24,29,33], but a further evaluation was necessary in view of the contradictory values reported by different authors for the molar absorbance coefficients of trinitrophenyl amino derivatives, the influence of liberated sulfite ions on the absorbance spectra and the possible reaction of TNBS with thiol groups. From the results (unpublished) we conclude that TNBS can be used for the quantitative determination of primary amino

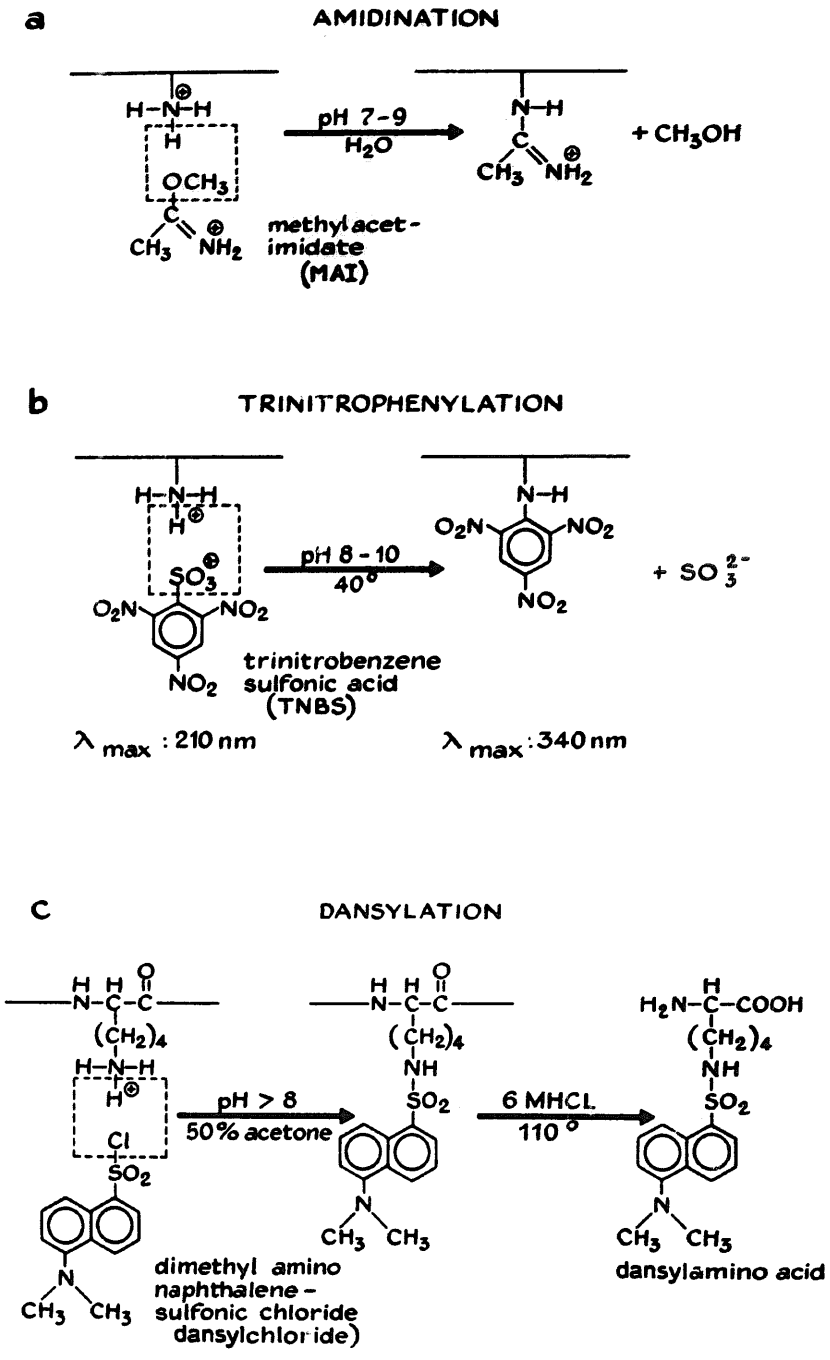


Fig. 1. Reaction equations of the amino group modifications described in this paper.

groups, using an $\varepsilon_{340}$, for the trinitrophenyl amino compounds, of $13100 \pm 400$.

Since TNBS, in contrast to methyl acetimidate introduces a large substituent and does not conserve the charge of the original amino groups, we also investigated the effect of modification of photoreceptor membranes by TNBS on some properties of rhodopsin.

For the identification of the primary amino groups left after various stages of modification, labeling with dansyl chloride was chosen for three reasons: the resulting dansyl amino compounds are strongly fluorescent, they are fairly resistant to hydrolysis and they may subsequently be identified by means of thin-layer chromatography

(Fig. 1c). Although dansyl chloride is not completely specific for amino groups, only the derivatives of amino groups and of the tyrosine hydroxyl group are stable towards acid hydrolysis[31].

*Primary amino groups present in rod outer segment membranes*

The total number of primary amino groups present in rod outer segment membranes was calculated from amino acid analysis as well as from reaction with TNBS and with dansyl chloride. The three values are in good agreement (Table I), averaging 420±10 nmoles/mg lyophilisate or 52±1 moles/mole of rhodopsin present, of which 16 are due to ε-amino groups of lysine, 27 to ethanolamine containing phospholipids and 9 to phosphatidylserine.

TABLE I

PRIMARY AMINO GROUPS, INCLUDING THE CHROMOPHORE BINDING GROUP, PRESENT PER MOLE OF RHODOPSIN IN ROD OUTER SEGMENT MEMBRANES

For experimental details see Methods. *n*, number of experiments.

| | | *Amino acid analysis* (*n*=5) | *Dansylation procedure* (*n*=4) | *Trinitrophenylation* (*n*=5) |
|---|---|---|---|---|
| Protein | Lysine | 16.3±0.2 | 16.4±0.8 | |
| Lipid | Ethanolamine | 26.7±0.6 | 27.9±1.0 | |
| | Serine | 8.8±0.2 | 8.2±0.6 | |
| | Total | 51.8±0.7 | 52.5±1.4 | 52.3±0.6 |

We do not confirm the observation of Reporter and Reed[34] that a large number of methylated basic residues (lysine, arginine, histidine) are present in rhodopsin. With our amino acid analyzing system, which does separate the methylated from the non-methylated residues, we do not detect any methylated histidine or methylated ε-amino-lysine. Moreover, TNBS and methyl acetimidate do not react under our conditions with the ε-methyl aminolysine group, while dansyl chloride yields ε-methyl-, ε-dansyl-aminolysine, which is much less stable towards acid hydrolysis than ε-dansylamino-lysine (Table III) and has a higher $R_F$ value (0.18) in Solvent system I. The number of amino groups shown in Table I, therefore, represent only primary amino groups.

*Amidination*

Amidination is performed on suspensions of membrane fragments. This has two advantages: first, detergents have a denaturing effect, resulting in loss of thermal stability, of regeneration capacity and of some enzymatic activities and in changes in the CD spectra; secondly the modified membranes can be recovered simply by sedimentation.

Due to hydrolysis, over 90% of the added methyl acetimidate is consumed within 30 min. Therefore, a large excess of the reagent is used and every half hour a new amount is added until modification is nearly complete. To avoid side effects caused by accumulation of hydrolysis products, the membranes are sedimented and resuspended in fresh buffer solution before each new addition of reagent.

When the amidination is carried out at pH 9 in a pH stat (20 °C) the results lack reproducibility. After amidination of 40–70% of the amino groups, the 500-nm absorbance decreases rather markedly, while the recombination capacity in some cases is abolished at an even lower percentage amidination.

Amidination at pH 7.5 in phosphate buffer gives reproducible results. Experiments carried out in the dark on non-illuminated samples yield 68% modification after one step (Fig. 2a). Subsequent steps increase this percentage by smaller amounts to a maximum of 98% after the fifth step. Thus two-thirds of the amino groups are easily accessible. The results also indicate that amidination up to 80% has little

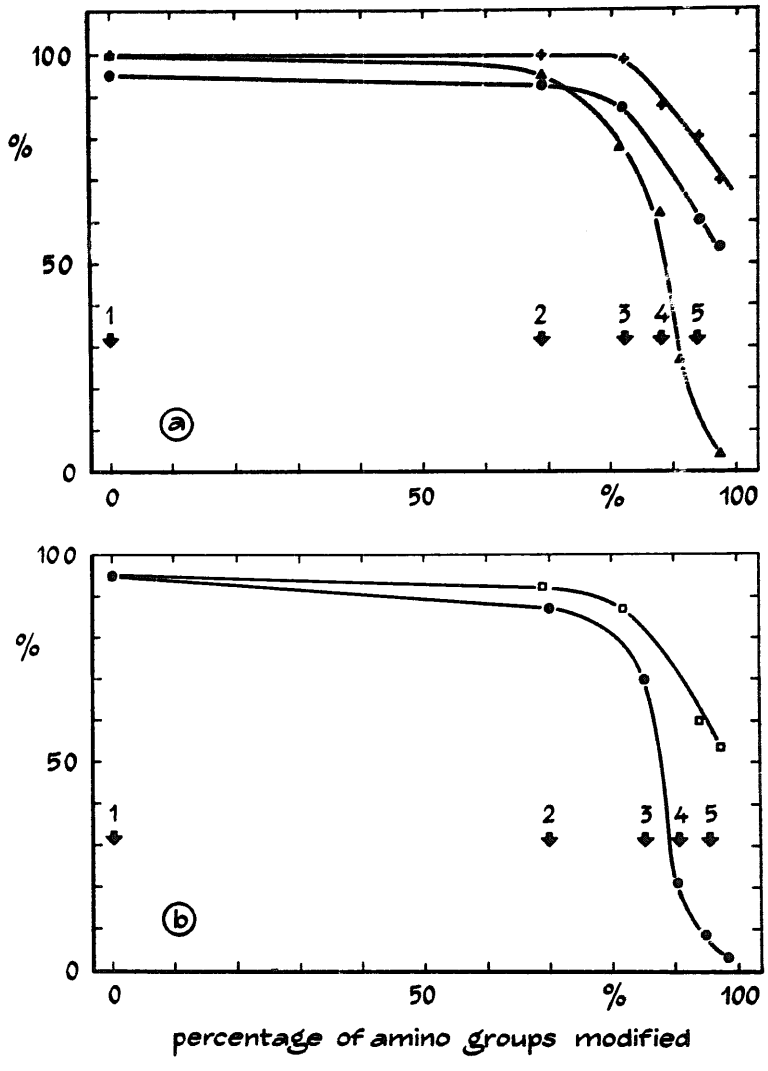


Fig. 2. Influence of amidination on some photoreceptor membrane properties. Amidination was performed as described under Materials and Methods. The arrows indicate the start of a new amidination step. The percentage of amidination was determined by reaction of the unmodified amino groups with TNBS. (a) Amidination performed in darkness. Effect of amidination on $\Delta A^{1}_{500}$ (+—+), recombination capacity (●—●) and retinol dehydrogenase activity (▲—▲). The values of non-amidinated controls are in each case taken as 100%. (b) Amidination performed in the light. Effect on recombination capacity (●—●). For comparison, the influence of amidination in darkness on the recombination capacity is also shown (□—□). The $\Delta A_{500}$ of the preparations before illumination is taken as 100%.

impact on photoreceptor membrane properties: the 500-nm absorbance and even the entire absorbance spectrum remain practically unchanged (Fig. 3), the recombination capacity is not significantly decreased, and the retinol dehydrogenase activity decreases by less than 25%.

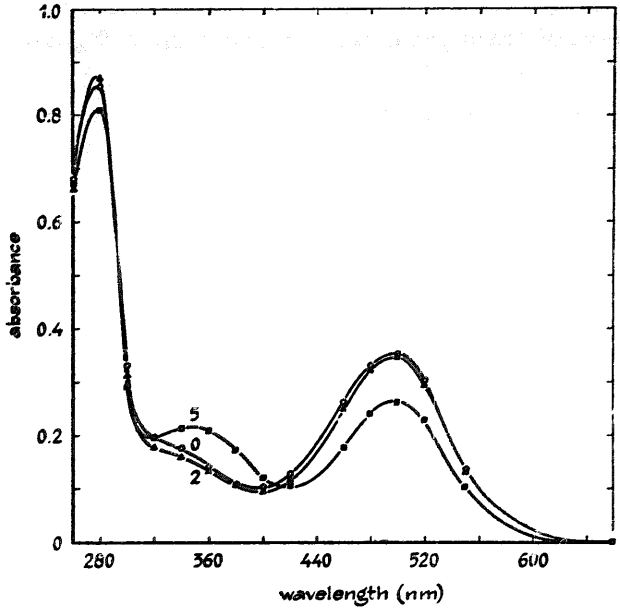


Fig. 3. Spectra of rod outer segment membranes, amidinated to various extents. The spectra were determined in 2% digitonin in 67 mM phosphate buffer (pH 6.5), containing 50 mM hydroxylamine and are recalculated to 1 mg lyophilisate per ml detergent solution. Spectra are shown for non-amidinated (0, o—o), twice amidinated (2, ▲—▲, 80% modification) and 5-fold amidinated material (5, ■—■, 97% modification). The peak at 355 nm in the third spectrum derives from retinylidene oxime; in the absence of hydroxylamine this peak has its maximal absorbance at 380 nm.

Upon further modification $\Delta A^1_{500}$ decreases gradually to about 70% of the original value, when amidination is nearly complete. Concurrent release of retinaldehyde is manifested by an increase in the absorbance around 380 nm (Fig. 3). The recombination capacity of the remaining rhodopsin also decreases gradually from about 90% to about 70%. The effect on retinol dehydrogenase activity is, however, drastic: after about 80% modification it declines rapidly and is practically abolished at 98% modification (Fig. 2).

After amidination rhodopsin remains stable at room temperature towards hydroxylamine (0.1 M), $NaBH_4$ and an aggressive detergent like cetyltrimethylammonium bromide.

When amidination is carried out in the light on previously illuminated membrane suspensions (15 min, filter OG2 Schott-Jena), the first two cycles yield comparable results to the unbleached samples (Fig. 2b). Upon further modification, however, the illuminated membranes behave quite differently. A rapid decrease in recombination capacity is observed, and at 97% modification this capacity is virtually abolished, while a variable loss of retinol dehydrogenase activity is detected. This light effect is even more directly demonstrated by the following experiment. A membrane suspension, amidinated to about 93% in the dark, was divided into two parts, one of

which was further modified to about 97% in the dark, while the other part was illuminated and amidinated to about the same extent in the light. The membrane fragments treated in the dark retained 72% of the original $\Delta A^1_{500}$ and 75% of their recombination capacity. The fragments treated in the light during the last cycle retained only 2% of the original $\Delta A^1_{500}$ and the recombination capacity was completely abolished.

The various washings during the modification procedure cause a 20–30% loss of material. However, no specific losses of protein or lipid occur since amino acid analysis, phosphorus determinations and retinaldehyde determinations indicate that no significant change takes place during modification in protein, phospholipid or retinaldehyde content, while the amino acid composition also remains the same. Moreover, $\Delta A^1_{500}$ and recombination capacity of the non-amidinated controls do not change during the entire procedure.

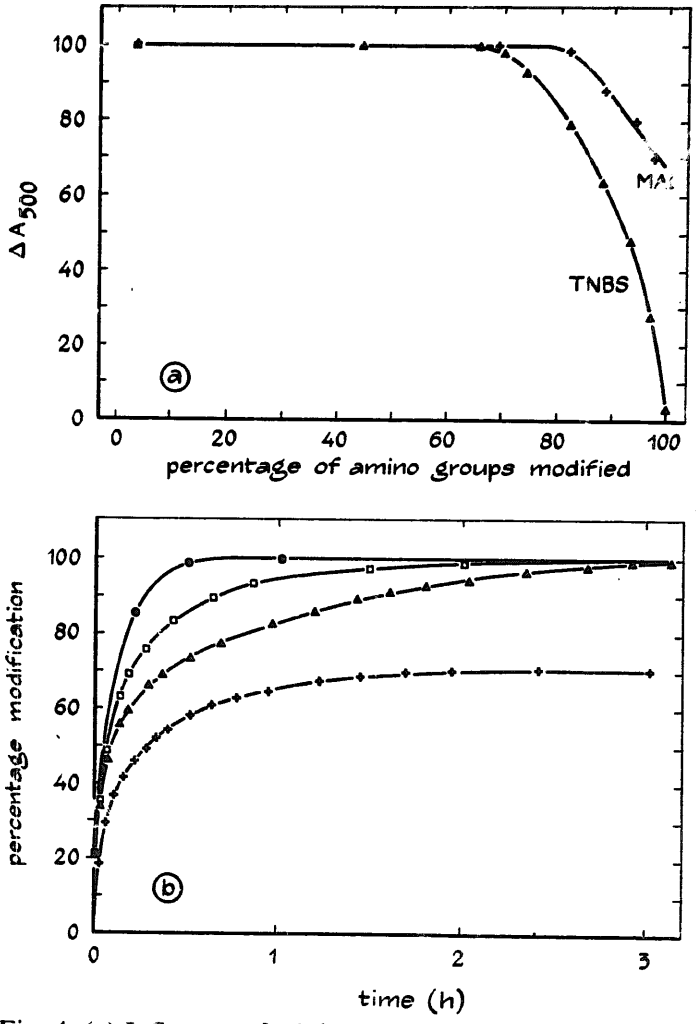


Fig. 4. (a) Influence of trinitrophenylation of rod outer segment membranes, suspended in 0.2 M bicarbonate (pH 8.0) in darkness at 40 °C, on the spectral integrity of rhodopsin expressed as $\Delta A_{500}$ (TNBS, ▲—▲). When performed at room temperature, the reaction virtually stops after 70% modification (compare Fig. 4b) without a decrease in $\Delta A_{500}$. For comparison, the influence of amidination in darkness on the spectral integrity is also shown (MAI, +—+). (b) Time course of the reaction of TNBS with rod outer segment membranes under various conditions: +—+, in darkness at 20 °C; ▲—▲, in darkness at 40 °C; □—□, in the light at 40 °C; ●—●, in the light at 40 °C after addition of up to 1% (w/w) Triton X-100.

Sodium dodecyl sulfate gel electrophoresis of the amidinated samples yields a pattern comparable to that of untreated membranes[2]: a fast-moving lipid band, a main protein band (opsin) with a molecular weight of about 38000 and some very weak protein bands of higher molecular weight.

*Trinitrophenylation*

The reaction of rod outer segment membranes, suspended in 0.2 M bicarbonate (pH 8), with a 15-fold excess of TNBS in the dark at 35–40 °C results in complete modification after about 3 h, concomitant with a complete loss of the 500-nm absorbance and of the recombination capacity (Fig. 4a). Illumination or addition of Triton X-100 accelerates the reaction (Fig. 4b). The 500-nm absorbance begins to decrease only after 65% of the amino groups have been modified. When trinitrophenylation is performed at 20 °C, modification virtually stops at about 70% without decrease in the 500-nm absorbance (Fig. 4b).

*Dansylation*

Dansylation is performed in 50% acetone[31]. This has the advantage that the membrane structure is denatured and the chromophore is released, so that all primary amino groups are readily accessible to the dansyl reagent.

A quantitative determination of the dansylated amino groups requires the following conditions: (a) dansylation should be virtually quantitative, (b) corrections should be applied for losses suffered during hydrolysis[30,31] and extraction from the thin-layer chromatographic plates, (c) the exact amount of protein originally present should be determined carefully, preferably by amino acid analysis.

Completeness of dansylation has been checked by amino acid analysis of dansylated membranes. Hydrolysis losses have been determined on the pure dansyl compounds (Table III). The loss of amino dansyl derivatives other than $\alpha$-derivatives appears to be mainly due to cleavage of the amide bond, generating dansylic acid and the original amino compound. In the $\alpha$-amino derivative *N*-adansylserine, however, cleavage of the C–N bond appears to occur almost as easily as cleavage of the amide bond (Table III), in agreement with earlier observations of Seiler[31].

When these corrections are applied to the amino acid analysis, the dansylation appears to be virtually quantitative (Table II). Table III also shows the relative amounts of dansyl compounds recovered after thin-layer chromatography and extraction. For the amounts used in our experiments, the loss of different dansyl derivatives is independent of the total amount applied. Correction for these losses may, therefore, be avoided by the use of reference substances.

Dansylation of unmodified membranes and subsequent hydrolysis yields apart from two cleavage products (dansyl amide and the blue fluorescing dansylic acid), only four dansyl compounds: *O*-dansyltyrosine, $\varepsilon$-aminodansyllysine, *N*-dansylserine and *N*-dansylethanolamine (Fig. 5). No dansyl derivatives of glucosamine and glycine were detected. Even though we have tried various systems, we do not observe any $\alpha$-aminodansyl derivative in amounts exceeding one-tenth the amount of rhodopsin except *N*-dansylserine. This is completely derived from phosphatidylserine, since it disappears upon enzymatic delipidation of the membranes[38,39] previous to dansylation (Fig. 5). In confirmation, amino acid analysis of dansylated membranes shows no

TABLE II

AMINO ACID ANALYSIS* OF ROD OUTER SEGMENT MEMBRANES BEFORE AND AFTER DANSYLATION

Results expressed per mole rhodopsin; 4 experiments.

| *Amino acid* | *Before* | *After* | *Δ*** (%) | *Amino acid* | *Before* | *After* | *Δ*** (%) | |
|---|---|---|---|---|---|---|---|---|
| Gly | 30.3 | 31.1 | +3±2 | Phe | 30.4 | 31.0 | + 2± 3 | |
| Asp | 30.4 | 29.1 | −4±4 | His | 7.2 | 7.2 | + 0± 3 | |
| Thr | 31.4 | 31.9 | +2±2 | Arg | 10.4 | 10.5 | + 1± 6 | |
| Glu | 37.7 | 38.3 | +2±2 | Glucosamine | 3.6 | 3.8 | + 7±10 | |
| Pro | 25.6 | 23.8 | −7±5 | Tyr | 18.2 | 5.4 | −70± 6 | |
| Ala | 35.3 | 34.9 | −1±1 | Lys | 15.9 | 4.0 (0.4) | −75± 2 | (−98±4) |
| Val | 28.5 | 27.9 | −2±4 | Ethanolamine | 27.1 | 4.9 (0.6) | −82± 3 | (−98±5) |
| Met | 12.7 | 13.9 | +9±7 | Ser (protein) | 22.0 | 22.4 | + 2± 2 | |
| Ile | 18.2 | 18.1 | −1±4 | Ser (lipid) | 8.9 | 2.3 (−0.1) | −74± 4 | (−101±8) |
| Leu | 35.0 | 34.7 | −1±1 | | | | | |

* Cys (partially) and Trp destroyed during hydrolysis.

** *Δ*, the differences with absolute standard error between the first two columns, in percentage. The values in parentheses are obtained after correction for the back formation of the amino acid from the corresponding dansyl derivative during hydrolysis (Table III).

TABLE III

LOSS OF DANSYL DERIVATIVES OBSERVED DURING ACID HYDROLYSIS (18 h) AND QUANTITATIVE THIN-LAYER CHROMATOGRAPHY

*n*, number of experiments.

| | *Recovery after acid hydrolysis (n=3)* | | *Recovery after thin-layer chromatography (n=4)* |
|---|---|---|---|
| | *Original compound* | *Dansyl amide* | |
| ε-*N*-Dansyllysine | 77±3% | Traces | 94±3% |
| ε-*N*-Methyl-ε-*N*-dansyllysine | 20–30% | N.D.* | N.D.* |
| *N*-Dansylserine | 49±3% | 24±2% | 92±3% |
| *N*-Dansylethanolamine | 84±3% | Traces | 95±4% |

* N.D., not determined.

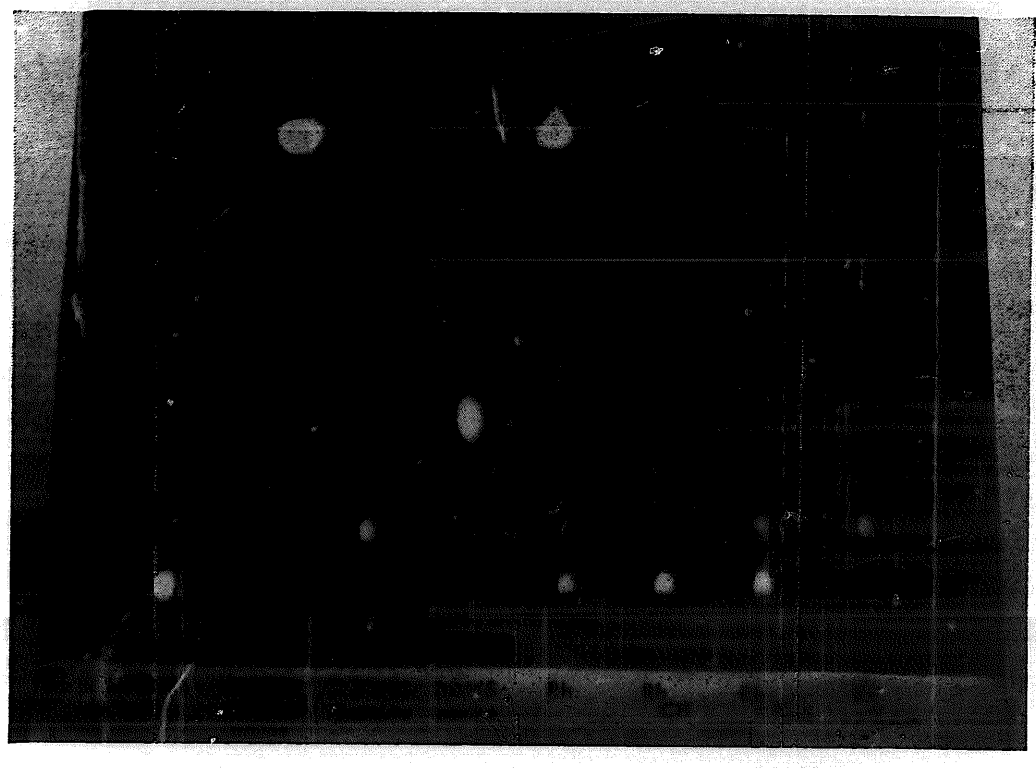

Fig. 5. Thin-layer chromatogram (silica gel G) obtained from hydrolysates of dansylated rod outer segment membranes, variously treated prior to dansylation. Solvent system: chloroform–methanol–acetic acid (15:4:1, by vol.). Excitation wavelength: 360 nm. The four spots on the left represent reference substances. *O*-Dansyltyrosine is contaminated with the blue fluorescing dansylic acid (1-dimethylaminonaphthalene-5-sulfonic acid; weak upper spot). The other dansyl derivatives produce yellow fluorescent spots. Rh, untreated membrane preparation; $Rh_{CH}$, membranes delipidated with phospholipase C and extracted with hexane; $Rh_{CHAS}$, membranes delipidated by treatments with phospholipase C, hexane, phospholipase A and serum albumin; $Rh_{97\%\ Am}$, extensively amidinated membranes; DANS, dansyl.

significant decrease in glucosamine, glycine or any other compound except lysine, ethanolamine, serine and tyrosine (Table II).

Upon calculation of the amino compounds present in untreated membranes, applying the corrections from Table III, good agreement is obtained with the results from amino acid analysis (Table I). Likewise, analysis of delipidated membranes confirms the results reported by Borggreven *et al.*[38] (Table IV).

Extensively dark-amidinated membranes show after dansylation and hydrolysis a substantial decrease in the amounts of *N*-dansyl derivatives (Fig. 6). As was to be expected, the amount of *O*-dansyltyrosine remains about the same. When membranes, illuminated before amidination, are dansylated, a comparable decrease in *N*-dansyl compounds is observed. However, dansylserine is still detected, while less *ε*-*N*-dansyllysine appears to be left. This is confirmed by quantitative determinations (Table IV). When the amidination is carried out in the dark, only the lysine/rhodopsin ratio exceeds 1.0. When it is carried out in the light, the number of primary *ε*-aminolysine groups is decreased, while surprisingly the number of non-amidinated phosphatidylserine residues is increased. Virtually complete modification can only be accomplished in five amidination steps when during illumination NADPH is added in

TABLE IV

PRIMARY AMINO GROUPS, INCLUDING THE CHROMOPHORE BINDING SITE, PRESENT PER MOLE OF RHODOPSIN IN ROD OUTER SEGMENT MEMBRANES, TREATED IN VARIOUS WAYS

The amino groups were determined by the dansylation procedure. *n*, number of experiments.

| | *Untreated* | *Delipidated ($Rh_{CH}$)*** | | *Amidinated* | | |
|---|---|---|---|---|---|---|
| | | *Our results (n=1)* | *ref. 38* | *Darkness (n=4)* | *After illumination* (n=3)* | *After illumination +NADPH addition (n=2)* |
| ε-Aminolysine | 16.4±0.8 | 17 ±2 | — | 1.4±0.2 | 0.5±0.1 | 0.4±0.3 |
| Ethanolamine | 27.9±1.0 | ≤0.3 | 0.3±0.1 | 0.4±0.2 | 0.5±0.3 | 0.2±0.2 |
| Serine | 8.2±0.6 | 1.8±0.6 | 1.9±0.3 | 0.2±0.2 | 0.9±0.3 | 0.1 |

* Calculated relative to the amount of rhodopsin present in membranes extensively amidinated in darkness.

** Membranes delipidated with phospholipase C and extracted with hexane.

order to permit reduction of the liberated retinaldehyde to retinol by the retinol dehydrogenase.

## DISCUSSION

### *General aspects of modification*

It has previously been demonstrated for several soluble proteins, that extensive amidination influences the conformation only to a moderate extent[24]. Our results show that this is also the case for the membrane protein rhodopsin. Amidination of about 99% of the primary membrane amino groups, *i.e.* 97% of the membrane protein lysine groups, has relatively little impact on the 500-nm absorbance and the recombination capacity. Of these two parameters the latter is rather sensitive to conformational changes, since it is, for instance, abolished by detergents.

The effects of reaction with TNBS differ strikingly from those of reaction with methyl acetimidate (Fig. 4a). It is primarily the loss of the positive charge of the amino groups reacting with TNBS, which causes a gradual disruption of the protein structure, leading to complete denaturation (see last section). These effects, however, manifest themselves only at elevated temperature (30–40 °C) and when more than 65% of the available amino groups have been modified. TNBS reacts much faster with the phospholipid amino groups than with the membrane protein lysine groups (unpublished). At temperatures below 20 °C, mainly phospholipids become modified and protein denaturation does not appear. This is similar to observations of Dratz and Schwartz[40] with the large aromatic reagent, fluorescein isothiocyanate.

Dansyl chloride appears to react slowly with the acetamidine group. Upon prolonged dansylation of amidinated membranes at 37 °C, increasing amounts of its hydrolysis product dansyl ethylamide appear on the chromatogram ($R_F$ 0.93 in System I and 0.45 in System II).

impact on photoreceptor membrane properties: the 500-nm absorbance and even the entire absorbance spectrum remain practically unchanged (Fig. 3), the recombination capacity is not significantly decreased, and the retinol dehydrogenase activity decreases by less than 25%.

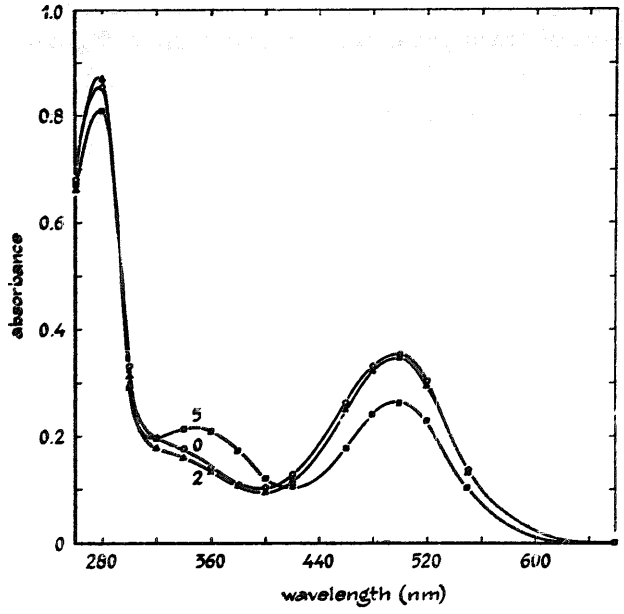


Fig. 3. Spectra of rod outer segment membranes, amidinated to various extents. The spectra were determined in 2% digitonin in 67 mM phosphate buffer (pH 6.5), containing 50 mM hydroxylamine and are recalculated to 1 mg lyophilisate per ml detergent solution. Spectra are shown for non-amidinated (0, ○—○), twice amidinated (2, ▲—▲, 80% modification) and 5-fold amidinated material (5, ■—■, 97% modification). The peak at 355 nm in the third spectrum derives from retinylidene oxime; in the absence of hydroxylamine this peak has its maximal absorbance at 380 nm.

Upon further modification $\Delta A^1_{500}$ decreases gradually to about 70% of the original value, when amidination is nearly complete. Concurrent release of retinaldehyde is manifested by an increase in the absorbance around 380 nm (Fig. 3). The recombination capacity of the remaining rhodopsin also decreases gradually from about 90% to about 70%. The effect on retinol dehydrogenase activity is, however, drastic: after about 80% modification it declines rapidly and is practically abolished at 98% modification (Fig. 2).

After amidination rhodopsin remains stable at room temperature towards hydroxylamine (0.1 M), $NaBH_4$ and an aggressive detergent like cetyltrimethylammonium bromide.

When amidination is carried out in the light on previously illuminated membrane suspensions (15 min, filter OG2 Schott-Jena), the first two cycles yield comparable results to the unbleached samples (Fig. 2b). Upon further modification, however, the illuminated membranes behave quite differently. A rapid decrease in recombination capacity is observed, and at 97% modification this capacity is virtually abolished, while a variable loss of retinol dehydrogenase activity is detected. This light effect is even more directly demonstrated by the following experiment. A membrane suspension, amidinated to about 93% in the dark, was divided into two parts, one of

the phosphatidylserine is closely associated with rhodopsin. The resistance to phospholipase C action of two phosphatidylserine molecules per mole rhodopsin[38] points in the same direction. Whether these play a physiological role in the "handling" of the liberated retinaldehyde remains uncertain. The relative inertness of phosphatidylserine in membranes, both chemically[42] and enzymatically[43], seems to be a rather general phenomenon and this phospholipid appears to be intimately associated also with other membrane proteins[44,45]. Recently, Cook *et al.*[46] suggested that in the nerve axon membrane the carboxyl group of phosphatidylserine is involved as a cation-exchange site in nervous excitation. An analogous situation might be present in visual excitation.

*Retinol dehydrogenase*

The particulate retinol dehydrogenase activity, which catalyses the reduction of retinaldehyde with NADPH as the preferred coenzyme[21,22], is very sensitive to amidination of the photoreceptor membrane (Fig. 2a). Since amidination has a relatively small influence on the integrity of rhodopsin and since added retinaldehyde can partly prevent the loss of retinol dehydrogenase activity, we conclude that the enzyme is inactivated by modification of a catalytic amino group. The involvement of such a group is not surprising in view of the easy formation of aldimine links by retinaldehyde, which would, therefore, be a suitable way to form an enzyme–substrate complex. This would also agree with the observed ability of retinol dehydrogenase to reduce not only free retinaldehyde, but also retinylidene imines[21]. In the latter case the retinaldehyde could transiminise rapidly from the carrier amino group under formation of the enzyme–substrate complex, so that the product formation remains the rate-determining step. One could further conceive of a transiminization of retinaldehyde away from the opsin binding site to the catalytic site of retinol dehydrogenase after illumination. By selective amidination we have indeed obtained evidence for such a transiminization, further support for which has been presented by reductive fixation of the chromophore to the photoreceptor membrane[47].

*Other modifying agents*

We are currently investigating the effects of other modifying agents: methylpiccoline imidate, dinitrofluorobenzene, succinic anhydride, nitrofluorobenzenesulfonate and some bifunctional imidates. In view of the differences in size and charge of the resulting substituents this might yield valuable information on structural features of the photoreceptor membrane. Preliminary observations with methylpiccoline imidate show that modification of more than 95% of the primary amino groups can be achieved with at least 80% of the rhodopsin remaining intact. This appears to indicate that the loss of positive charge on the amino groups, such as occurs with TNBS, has more effect on the structural integrity of rhodopsin than mere introduction of a large substituent, such as with methylpiccoline imidate.

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Sodium dodecyl sulfate gel electrophoresis of the amidinated samples yields a pattern comparable to that of untreated membranes[2]: a fast-moving lipid band, a main protein band (opsin) with a molecular weight of about 38000 and some very weak protein bands of higher molecular weight.

*Trinitrophenylation*

The reaction of rod outer segment membranes, suspended in 0.2 M bicarbonate (pH 8), with a 15-fold excess of TNBS in the dark at 35–40 °C results in complete modification after about 3 h, concomitant with a complete loss of the 500-nm absorbance and of the recombination capacity (Fig. 4a). Illumination or addition of Triton X-100 accelerates the reaction (Fig. 4b). The 500-nm absorbance begins to decrease only after 65% of the amino groups have been modified. When trinitrophenylation is performed at 20 °C, modification virtually stops at about 70% without decrease in the 500-nm absorbance (Fig. 4b).

*Dansylation*

Dansylation is performed in 50% acetone[31]. This has the advantage that the membrane structure is denatured and the chromophore is released, so that all primary amino groups are readily accessible to the dansyl reagent.

A quantitative determination of the dansylated amino groups requires the following conditions: (a) dansylation should be virtually quantitative, (b) corrections should be applied for losses suffered during hydrolysis[30,31] and extraction from the thin-layer chromatographic plates, (c) the exact amount of protein originally present should be determined carefully, preferably by amino acid analysis.

Completeness of dansylation has been checked by amino acid analysis of dansylated membranes. Hydrolysis losses have been determined on the pure dansyl compounds (Table III). The loss of amino dansyl derivatives other than $\alpha$-derivatives appears to be mainly due to cleavage of the amide bond, generating dansylic acid and the original amino compound. In the $\alpha$-amino derivative *N*-adansylserine, however, cleavage of the C–N bond appears to occur almost as easily as cleavage of the amide bond (Table III), in agreement with earlier observations of Seiler[31].

When these corrections are applied to the amino acid analysis, the dansylation appears to be virtually quantitative (Table II). Table III also shows the relative amounts of dansyl compounds recovered after thin-layer chromatography and extraction. For the amounts used in our experiments, the loss of different dansyl derivatives is independent of the total amount applied. Correction for these losses may, therefore, be avoided by the use of reference substances.

Dansylation of unmodified membranes and subsequent hydrolysis yields apart from two cleavage products (dansyl amide and the blue fluorescing dansylic acid), only four dansyl compounds: *O*-dansyltyrosine, $\varepsilon$-aminodansyllysine, *N*-dansylserine and *N*-dansylethanolamine (Fig. 5). No dansyl derivatives of glucosamine and glycine were detected. Even though we have tried various systems, we do not observe any $\alpha$-aminodansyl derivative in amounts exceeding one-tenth the amount of rhodopsin except *N*-dansylserine. This is completely derived from phosphatidylserine, since it disappears upon enzymatic delipidation of the membranes[38,39] previous to dansylation (Fig. 5). In confirmation, amino acid analysis of dansylated membranes shows no

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