Table 1. The amounts of α_T, β_T, and rhodopsin (Rh) measured by laser densitomer scans of Coomassie blue–stained gels. Values are expressed as the mean ± SEM ($n = 6$).

	α_T/Rh	β_T/Rh	$\alpha_T/\alpha_T + \beta_T$
Light	0.43 ± 0.04	0.50 ± 0.05	0.46 ± 0.02
Dark	0.45 ± 0.04	0.56 ± 0.06	0.44 ± 0.02

major contaminants. The identification of rat transducin subunits was unambiguous (*10*) by (i) comparison with purified bovine transducin standard, (ii) total elution from ROS washed with 1 m*M* GTP, and (iii) cross-reactivity with the antiserum to transducin. In addition, loss of transducin due to proteolysis was unlikely as shown by the presence of only the 37,39-kD transducin α, β doublet on immunoblots.

Quantification of transducin:rhodopsin ratios for purified rat ROS demonstrated that the concentration of α and β transducin subunits did not vary during the daily light:dark cycle (Fig. 1 and Table 1). In addition, no soluble pool of transducin was preferentially lost during preparation of dark- or light-adapted ROS. In contrast, the major dark-soluble ROS protein, 48K (*11*), was reduced in concentration in ROS membranes prepared from dark-adapted retinas (Fig. 1, b through d).

Thus, massive depletion of transducin polypeptides from ROS did not occur at any time during the daily light-dark cycle. Explanations other than subunit translocation must account for the two- to fourfold differences (*1*) in light-dark transducin immunoreactivity within the ROS. One possibility is that transducin, like rhodopsin, undergoes light-induced (*12*) covalent modifications. Other possibilities include conformational changes in α_T or masking of antigenic sites on transducin by tight binding to rhodopsin in light-adapted ROS.

To test this we repeated the tissue-staining experiments of Brann and Cohen (*1*) on light and dark retinas under two different conditions: (i) with formaldehyde fixation to preserve the native conformation of transducin and (ii) with cold acetone fixation to denature the transducin in the tissue section. The two conditions produced very different results (Fig. 2). Formaldehyde fixation, as shown originally by Brann and Cohen, resulted in light-dark differences in α_T staining. In contrast, a more equal light-dark staining of ROS in acetone-fixed sections suggests the relevant transducin epitopes are unmasked by denaturation. Therefore, both biochemical and immunocytochemical results support the conclusion that transducin is present in both light- and dark-adapted ROS in comparable amounts, but that at least one antigenic site on the native α_T molecule is masked under light-adapted conditions.

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2. M. Pines, P. Gierschik, G. Milligan, W. Klee, A. Spiegel, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 4095 (1985).
3. Rat retinas were dissected under room lights or under dim red light and placed in buffered sucrose (34% sucrose, 65 m*M* NaCl, 2 m*M* $MgCl_2$, 5 m*M* tris acetate, *p*H 7.4) at 4°C. The retina suspension was gently shaken for 1 min and large retina pieces were removed by centrifugation (5 min, 4500*g*). The ROS-containing supernatant was collected and diluted with two volumes of TBS (10 m*M* tris, 0.15*M* NaCl, *p*H 7.4), and centrifuged (10 min, 4500*g*). The supernatant was removed and assayed for soluble transducin. The ROS pellet was resuspended in 50 μl of TBS and sampled for light microscopy. The remainder was solubilized in SDS sample buffer for analysis by SDS–polyacrylamide gel electrophoresis (*5*).
4. The 500-nm and 280-nm absorbance of dark-adapted ROS was determined by solubilizing the ROS in 1% Ammonyx LO (Onyx Chemical Co.) and measuring peak heights before and after complete bleaching and used to estimate the purity and intactness of ROS preparations [H. G. Smith, Jr., G. W. Stubbs, B. J. Litman, *Exp. Eye Res.* **20**, 211 (1975)].
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8. Quantification of rhodopsin and α_T and β_T was performed on gel lanes loaded with similar amounts of protein (10 to 20 μg). However, the ratios of rhodopsin to transducin were also checked for selected samples over a threefold range of protein concentration (7 to 20 μg) and were independent of the amount of total protein loaded. Transducin subunits were normalized to rhodopsin values rather than to total protein because rhodopsin, an integral membrane protein, does not change concentration in the ROS during the light-dark cycle. The small amount of rhodopsin dimer in both dark- and light-adapted samples was not included in the quantitation.
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10. Two major sources of error in quantifying ROS transducin can be excluded: (i) Contribution of transducin from retinal cells other than rod photoreceptors. Within the retina, transducin is found only in photoreceptors. [G. B. Grunwald, P. Gierschik, M. Nirenberg, A Spiegel, *Science* **231**, 856 (1986); C. L. Lerea, D. E. Somers, J. B. Hurley, I. B. Klock, A. H. Bunt-Milam, *ibid.* **234**, 77 (1986)]. (ii) Contributions from "contaminating" G proteins with α subunits identical in mobility to transducin (that is, G_0). The ratio of α_0:α_T in the ROS is less than 0.1% [S. M. Mumby, R. A. Kahn, D. R. Manning, A. G. Gilman, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 265 (1986)].
11. 48K (S-antigen, arrestin) was identified in these preparations by its characteristic solubility in the dark and from immunoblots of dark- and light-adapted preparations with a previously characterized antiserum to 48K [N. Mangini, D. Roof, W. Baehr, M. Applebury, D. Pepperberg, *Invest. Ophthalmol. Vis. Sci.* **25**, 112 (1984)].
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13. We thank R. Swanson and B. Knox for helpful discussions on the quantification of ROS proteins, A. Speigel for antiserum to transducin, and M. Adamian for help in the preparation of the figures. Supported by NIH grants EY06514 (DJR) and EY05790 (CAH) and a grant from the Retinitis Pigmentosa Foundation.

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Response: Roof and Heth report a disagreement between subcellular fractionation and immunocytochemical data concerning the amount of the alpha subunit of transducin (α_T) within rod outer segments (ROS). As we previously reported (*1*), they observe that, under certain fixation conditions, the α_T immunoreactivity of ROS is less during the day then at night. On the other hand, when they isolated ROS and measure α_T content by scanning SDS-polyacrylamide gels, α_T levels appear constant. On the basis of this disagreement, the authors interpret the data as showing that the amounts of α_T do not change and that light induces a masking of antigenic sites on α_T. I believe this conclusion is unlikely to be correct. First, the observed changes in α_T immunoreactivity are difficult to explain in terms of antigen masking. Second, the presented measurements of α_T levels in isolated ROS are confounded by technical limitations. These limitations are reinforced by the recent publication of a report by Philp *et al.* (*2*) which demonstrates that the α_T levels in isolated ROS change as predicted by immunocytochemical data.

Epitope masking is an unlikely explanation of the immunocytochemical data because at least three spatially separated epitopes would have to be involved. The antisera to α_T used in our study recognize two epitopes, one in the NH_2-terminal region of the protein and one within a central segment (*3*). Further, the immunocytochemical observations were confirmed with a second antibody that is directed to the COOH-terminus of α_T. Even more difficult to explain with such a model are the simultaneous and reciprocal changes in immunoreactivity in the rod inner segments (RIS). That is, even if light were able to simultaneously block three antigenic sites on α_T in the ROS, what is the mechanism of reciprocal changes in immunoreactivity in the RIS? In support of their epitope-masking hypothesis, Roof and Heth present a comparison of immunocytochemical data in which two fixation procedures were used. When tissue was fixed with acetone, a less marked change in immunoreactivity was observed than

when formaldehyde was used. They suggest that acetone denatures the protein while formaldehyde keeps it in a native conformation where the epitopes are masked. No data are presented to support this suggestion; in fact, an analysis of the presented photographs suggests that the acetone-treated sections have more nonspecific immunoreactivity, as evidenced by immunoreactivity in the inner nuclear layer, a region with no α_T.

The conclusions of Roof and Heth are largely based on the data presented in their figure 1, and on its quantification as presented in their table 1. As with any negative data, a major question is, What magnitude of change would be detectable with the methods used? Densitometric analysis of proteins run on SDS-polyacrylamide gels is technically problematic (particularly when the data are standardized in terms of the ratio of the optical density of one protein to another that is present at much higher concentrations). That is, due to saturation of optical signals, protein concentration is related in a linear fashion to optical density only over a very limited range of concentrations. Because there are much larger amounts of opsin than of α_T, the amount of opsin may be underestimated, and the ratio of the two proteins may not reflect the true α_T concentration. Another problem with these data, which is alluded to by Roof and Heth, is that a pool of α_T may be selectively lost during the isolation of the ROS. If α_T is transported in bulk, then it is likely that a major portion is only loosely associated, if at all, with the disk membranes, α_T would therefore be easily lost during isolation procedures. Unless the amount of this lost protein is included in the quantification, a greater loss at night could cause underestimation of the amount of α_T in the dark and obscure an increase.

In conclusion, I believe our original conclusion stands: α_T moves in bulk from RIS to ROS as a function of the light-dark cycle. Regarding the hypothesis that these changes are related to dark adaptation, translocation of α_T has been induced directly by changes in illumination; and the time course of these changes are consistent with the time course of dark adaptation (*2*).

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