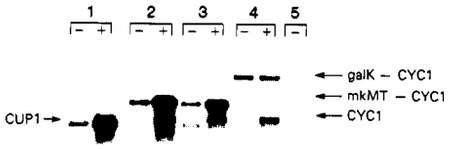

Fig. 4. Analysis of RNA in *cup1*$^{\Delta}$ transformants. Transformants were grown to mid-logarithmic phase in synthetic dextrose medium under conditions to select for plasmid maintenance. One half of each culture was then induced with 75 μM $CuSO_4$ for 60 minutes at 30°C. Cells were harvested and RNA isolated as previously described (*5*). Polyadenylated RNA was enriched by oligo(dT)-cellulose chromatography. One microgram of each RNA sample was subjected to electrophoresis on a 1.5 percent formaldehyde-agarose gel, transferred to nitrocellulose, and hybridized with either a ^{32}P-labeled nick-translated *CUP1* 1.1-kilobase Xba I/Kpn I fragment (section 1) or a *CYC1* 597-base pair Eco RI/Hind III fragment (sections 2 to 5). Transcripts originating at the *CUP1* promoter in the constructs described in Fig. 1 share the common *CYC1* region. Autoradiography was carried out at −70°C with an intensifying screen. The RNA samples were purified from the following plasmid transformants of 55.6B (*cup1*$^{\Delta}$): YEp336 (section 1); HYM1 (section 2); HYM2 (section 3); HCG (section 4); untransformed BR10 (section 5). The symbols − or + indicate growth in the absence or presence of 75 μM $CuSO_4$, respectively. CUP1 indicates the *CUP1* mRNA (500 nucleotides); CYC1 indicates the chromosomal *CYC1* transcript (630 nucleotides); mkMT-CYC1 indicates the monkey MT-I or MT-II *CUP1–CYC1* transcripts (approximately 800 nucleotides); and galK-CYC1 indicates the *E. coli* galactokinase–*CYC1* mRNA (1700 nucleotides). The increase in *CYC1* mRNA was not reproducible in different RNA analyses and the *CYC1* promoter is not inducible by copper (*5*).

MT isoforms reestablish transcriptional regulation from the *CUP1* promoter by repressing basal transcription to a level similar to that mediated by authentic yeast copperthionein. We have suggested two possible mechanisms for autoregulation of *CUP1* (*5*). (i) The *CUP1* gene product might act as a direct or indirect transcriptional repressor. The present data do not support this idea, because mammalian MT and yeast copperthionein share little primary sequence homology except for a stretch of six amino acids (*3, 4*) and because the upstream heavy metal control sequences of the mammalian and yeast genes are completely divergent (*3, 9–11*). Furthermore, purified yeast copperthionein and apothionein (*8*) exhibit no obvious DNA binding activity. (ii) Copperthionein might be feedback-regulated simply by sequestering intracellular copper that would otherwise be available to activate putative copper-dependent transcriptional factors. Our observation that mammalian MT can replace the autoregulatory function of yeast copperthionein supports this idea since mammalian proteins can chelate copper. Thus, our experiments suggest that yeast copperthionein, mammalian MT, and probably all other MT-like proteins from eukaryotes constitute a superfamily of proteins with closely related functions.

REFERENCES AND NOTES

1. J. H. R. Kagi and M. Nordberg, *Metallothionein* (Birkhauser Verlag, Basel, 1979).
2. S. Fogel and J. W. Welch, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 5342 (1982).
3. M. Karin *et al.*, *ibid.* **81**, 337 (1984).
4. T. R. Butt *et al.*, *ibid.*, p. 3332.
5. D. H. Hamer, D. J. Thiele, J. F. Lemontt, *Science* **228**, 685 (1985).
6. C. J. Schmidt and D. H. Hamer, *Gene* **24**, 137 (1983).
7. T. R. Butt, E. Sternberg, J. Herd, S. T. Crooke, *ibid.* **27**, 23 (1984).
8. D. R. Winge, K. B. Nielson, N. R. Gray, D. H. Hamer, *J. Biol. Chem.* **260**, 14464 (1985).
9. M. Karin *et al.*, *Nature (London)* **308**, 513 (1984).
10. A. D. Carter *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 7392 (1984).
11. G. W. Stuart *et al.*, *ibid.*, p. 7318.

12. We thank C. Schmidt for the plasmids containing the mkMT cDNA's and R. DiLauro and C. Schmidt for comments on the manuscript. D.J.T. was supported by a Damon Runyon–Walter Winchell Cancer Fund Fellowship, DRG-843.

20 May 1985; accepted 28 October 1985

Detection of α-Transducin in Retinal Rods But Not Cones

GERALD B. GRUNWALD,* PETER GIERSCHIK,† MARSHALL NIRENBERG, ALLEN SPIEGEL

The distribution in chicken retina of the α subunit of transducin, the guanine nucleotide–binding protein that couples light-dependent activation of rhodopsin with activation of guanosine 3′,5′-monophosphate phosphodiesterase, was determined with the aid of a specific antiserum. α-Transducin was found in rod photoreceptor cells but was not detected in cones. These results show that rods and cones differ with respect to α-transducin content and suggest that the processes of phototransduction may differ correspondingly in rods and cones.

LIGHT IS CONVERTED INTO SYNAPTIC membrane potentials in rod photoreceptor cells by a sequence of reactions that begins with the photoisomerization of the retinal moiety of rhodopsin. This results in the activation of cyclic guanosine monophosphate (cyclic GMP) phosphodiesterase molecules that catalyze the hydrolysis of cyclic GMP, thereby reducing the permeability of rod outer segment plasma membranes to Na^+ ions. Transducin, a guanosine triphosphate (GTP)–binding protein that indirectly couples rhodopsin-mediated effects of light with activation of cyclic GMP phosphodiesterase, plays a central role in the response of rod photoreceptor cells to light (*1–3*). Less is known about the mechanism of phototransduction in cones. There are indications that the exon-intron segments of cone opsin genes are like those of rhodopsin but that the amino acid sequences of cone opsin proteins have diverged somewhat from those of rhodopsin (*4, 5*). Evidence for (*6*) and against (*7*) cyclic GMP phosphodiesterase activity in cones has been reported, and light-dependent regulation of cyclic adenosine monophosphate (cyclic AMP), rather than cyclic GMP, has been proposed for cones (*8*). However, evidence for a light-dependent activation of cyclic GMP phosphodiesterase in cones also has been reported (*9, 10*).

A cyclic GMP–sensitive conductance was found in outer segment membranes of cones as well as rods (*11, 12*). These results suggest that cyclic GMP is the internal transmitter for phototransduction in both types of photoreceptors. The existence of a guanine nucleotide–binding protein that couples cone photopigments to cyclic GMP phosphodiesterase has been postulated (*13*), but there are no data on its identity or relation to rod outer segment transducin.

We conducted immunocytochemical studies on the distribution of α-transducin in chicken retina and on the expression of α-transducin during retina development.

G. B. Grunwald and M. Nirenberg, Laboratory of Biochemical Genetics, National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, MD 20892.
P. Gierschik and A. Spiegel, Metabolic Diseases Branch, National Institute of Arthritis, Diabetes, Digestive and Kidney Disease, National Institutes of Health, Bethesda, MD 20892.

*Present address: Department of Anatomy, Jefferson Medical College, Philadelphia, PA 19107.
†Present address: Pharmakologisches Institut, Universität Heidelberg, Federal Republic of Germany.

Transducin, like the related GTP-binding proteins N_s, N_i, and N_o, consists of three subunits (*2, 3*). The α subunit of transducin (M_r, 39,000) differs from the α subunits of N_s, N_i, and N_o. The β subunit (M_r, 35,000) of transducin is thought to be similar, or identical, to the β subunits of N_s, N_i, and N_o (*14–16*); and there is immunochemical evidence that the γ subunit of transducin (M_r, 8400) (*17*) differs from the γ subunits of N_s and N_i (*15*). We used antiserum GI-2 obtained from a rabbit that had been immunized with α-transducin subunits purified from bovine rod outer segments. The antibodies bind to α-transducin but do not cross-react with the α subunits of N_s, N_i, or N_o or with the β and γ subunits of transducin (*15, 16*).

The specificity of antiserum GI-2 is shown in Fig. 1. Protein from outer segments of bovine retina photoreceptors, purified transducin, or subunits of transducin were fractionated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and total protein was visualized with Coomassie blue, whereas proteins immunoreactive with antiserum GI-2 were detected by immunoblotting as described in the legend to Fig. 1. Antiserum GI-2 bound to the α subunit of transducin (Fig. 1B, lanes 6, 7, and 8) and did not bind to other proteins in bovine rod outer segments (Fig. 1B, lane 5). Antiserum GI-2 also bound only to one band of protein from chicken retina (lane 11) that had the same electrophoretic mobility as α-transducin from bovine retina (lane 12). Proteins from the entire retina were fractionated (lane 11); hence the intensity of the α-transducin band in lane 11 is relatively low compared to that of α-transducin in purified membranes of bovine rod outer segments (lane 5) or purified α-transducin (lane 7).

The distribution of antigen recognized by antiserum GI-2 in unfixed, frozen sections of chicken, bovine, rhesus monkey, and human retinas was determined by indirect immunofluorescence. Antigen was detected only in the photoreceptor layer of retina from each species tested. Additional studies were performed with embryonic and adult chicken retinas because retina development has been well characterized in chick embryos and because photoreceptor outer segments are large and cones are abundant (*18–21*). Elongating photoreceptor outer segment buds first appear in chicken retina on day 15 of embryonic development (*20, 21*). α-Transducin was not detected in sections of retina from chick embryos on days 7, 10, 13, or 16 of development, but was detected on day 18 in the photoreceptor cell layer only (Fig. 2A). Electroretinogram activity also appears on day 18 of chick embryo retinal development (*18*). The abundance of α-transducin increased between day 18 of embryonic development and the day of hatching (day 21) (Fig. 2B).

Two classes of photoreceptor cells can be seen in adult retinas (Fig. 2, C through I). Some photoreceptors contain abundant α-transducin, whereas others contain little or no α-transducin. Photoreceptor cells that were stained by the antiserum contained α-transducin in the outer segments, inner segments, and cell soma. Rod photoreceptors have large-diameter outer segments that taper to small-diameter inner segments lacking oil droplets. The thin inner segments and absence of oil droplets indicate that the cells that contain abundant α-transducin are rod photoreceptors. Between the brightly fluorescent rod photoreceptor cells there are unstained photoreceptor cells with inner segments of large diameter that contain oil droplets and large paraboloids. This suggests that the cells with little or no α-transducin are cones. The retina section shown in Fig. 2D was incubated with normal rabbit serum, rather than with antiserum GI-2, to show the autofluorescence of the oil droplets and nonspecific fluorescence of the cells.

Photomicrographs (Fig. 2) were obtained without the barrier filter (Zeiss LP520) that usually was used for epifluorescence microscopy so that cones could be identified by the autofluorescence of the oil droplets. When the barrier filter was used for microscopy, the specific fluorescence of rods was high, but no specific fluorescence was detected in cones (not shown). The difference between rods and cones with respect to α-transducin content can be seen in the photomicrograph obtained at high magnification in Fig. 2E. The difference between rod and cone fluorescence also is apparent in oblique sections of retina (Fig. 2F). Oil droplets of cones are shown at the edges of an oblique section of retina cut through the distal portion of the inner segments. The center of the section cuts through proximal portions of the inner segments of cones, which are larger in diameter than rod inner segments. Again, antiserum GI-2 was found to bind to rod but not to cone photoreceptors.

The specificity of antibody GI-2 for α-transducin was shown by incubation of

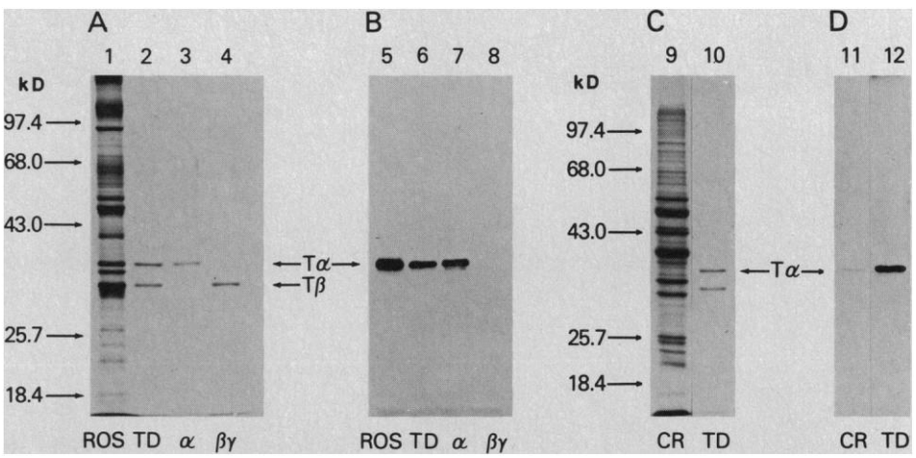

Fig. 1. SDS-PAGE and immunoblotting of bovine and chick retinal proteins; (A and B) (lanes 1 and 5) 50 μg of bovine rod outer segment protein; (lanes 2 and 6) 2 μg of purified bovine transducin (α, β, and γ subunits); (lanes 3 and 7) 1 μg of bovine α-transducin; (lanes 4 and 8) 1 μg of β and γ subunits of bovine transducin were separated by SDS-PAGE (10 percent gel) and stained with Coomassie blue (A) or immunoblotted (B) with antiserum GI-2, a rabbit antiserum raised against purified bovine α-transducin; (C and D) 150 μg of protein from whole chicken retina (lanes 9 and 11) or 2 μg of purified bovine transducin (lanes 10 and 12) were subjected to SDS-PAGE (10 percent gel) and either stained with Coomassie blue (C) or immunoblotted (D) with GI-2. Bovine rod outer segment membrane preparation, purification of transducin, SDS-PAGE, and immunoblotting were performed as in (*15, 16*). Briefly, proteins separated by SDS-PAGE were transferred to nitrocellulose paper electrophoretically; the paper was incubated with 3 percent gelatin (weight to volume) in tris-buffered saline (TBS) (20 m*M* tris-HCl, *p*H 7.5, and 500 m*M* NaCl) for 1 hour at room temperature to block nonspecific protein binding and then incubated overnight with a 100-fold dilution of antiserum GI-2 in 1 percent gelatin plus TBS. After the paper was washed with a solution of TBS containing 0.05 percent (by volume) Tween-20, the filter was incubated for 2 hours at room temperature with 1.5 μg of affinity-purified goat antibody to rabbit immunoglobulin conjugated to peroxidase (purchased from Kierkegaard & Perry) per milliliter of TBS supplemented with 1 percent gelatin. The filter was washed again with Tween-20–TBS solution and color-developed with 4-chloronaphthol. Separation and purification of transducin subunits was performed as in (*15, 16*). Whole retinas from adult chickens were homogenized in SDS-PAGE sample buffer. The positions of molecular weight standards (purchased from BRL; prestained high molecular weight) also are shown. Arrows indicate the α or β subunits of transducin.

the antiserum with excess purified bovine α-transducin or purified bovine β and γ subunits before the antibody was incubated with sections of chicken retina. Specific immunofluorescence of rods, but not cones, is shown in Fig. 2G (antiserum GI-2 incubated first with buffer and then with retina). In contrast, little or no specific immunofluorescence was observed when the antiserum was incubated with excess purified bovine α-transducin before incubation with the retina (Fig. 2H). Incubation of the antiserum with purified β and γ subunits of transducin before incubation with the retina section did not block specific staining of rod photoreceptors (Fig. 2I). These results, together with the immunoblot results, indicate that antiserum GI-2 binds to α-transducin of rods in chicken retina, whereas little or no antiserum to α-transducin binds to cones.

This result is surprising because, although rods and cones differ in various ways, a common mechanism of signal transduction mediated by transducin might be expected. One difference between rods and cones is that rods function primarily in dim light and can detect a single photon of light (*22, 23*). In contrast, cones function mainly in bright light. Thus, light-dependent reactions in rods may require greater amplification than those in cones. Our results show that rods and cones differ in α-transducin content. Possible explanations are that the phototransduction mechanism in cones requires relatively few molecules of α-transducin compared to that in rods, that cones express a species of α-transducin that is not detected by antiserum GI-2, or that rods and cones utilize quite different phototransduction mechanisms.

Complementary DNA (cDNA) for bovine retina α-transducin was cloned recently

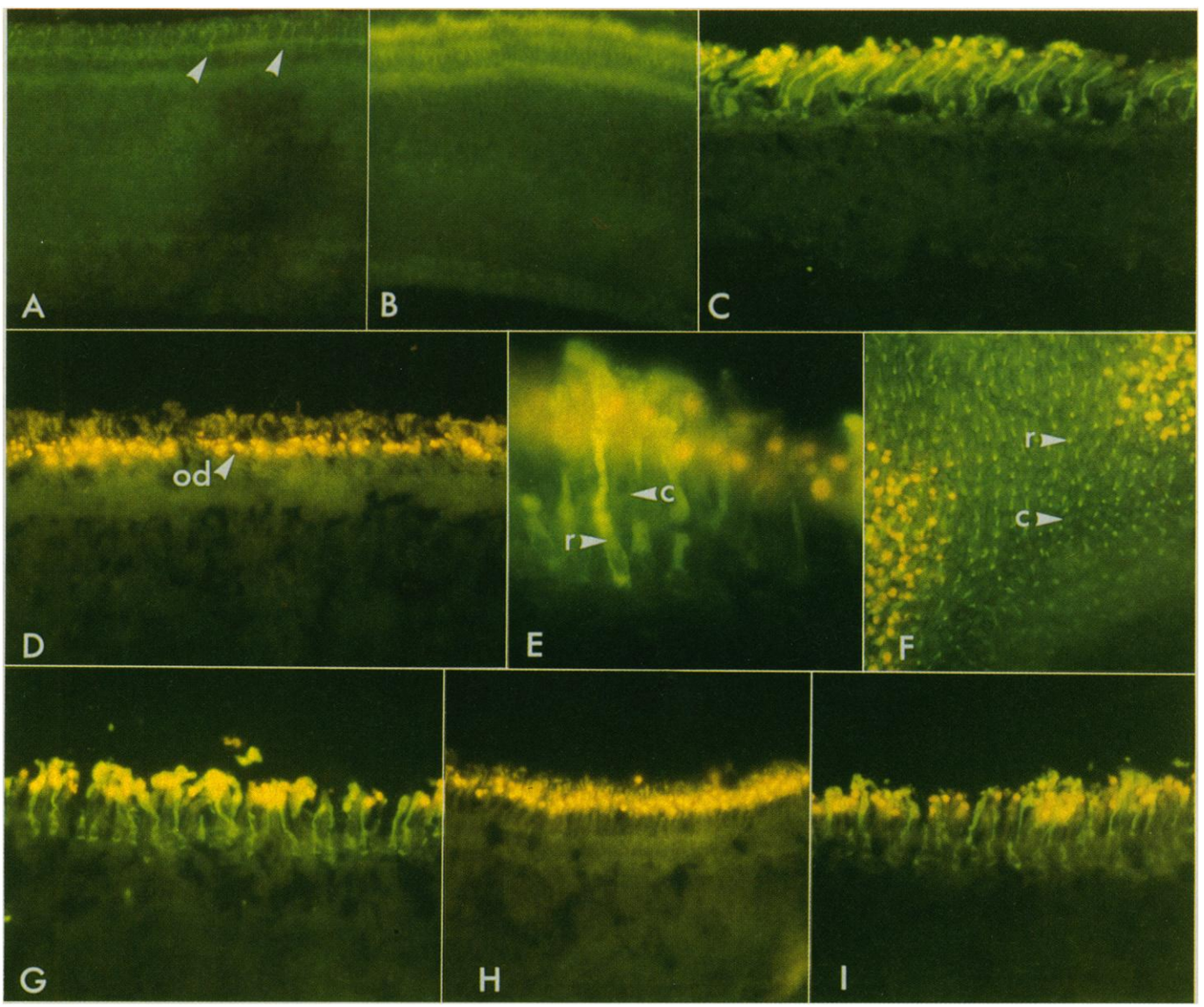

Fig. 2. Immunocytochemical localization of α-transducin in chicken embryo and adult retinas. Fresh, unfixed retinas from (A) 18-day chick embryos, (B) newly hatched chicks, or (C to I) adults were dissected, embedded, sectioned on a cryostat, and stained with antiserum GI-2 by indirect immunocytochemistry as described (*29*), except that a barrier filter was not used so that the yellow-orange autofluorescence of oil droplets could be used to identify cone photoreceptors. Antiserum GI-2 was used at a dilution of 1:200 (A to C and E and F) or 1:1000 (G to I). Normal rabbit serum was used at 1:200 dilution (D). Diluted antiserum was incubated first either without added protein (G), with 0.5 μg of purified α-transducin from bovine rod outer segments (H), or with 0.5 μg of purified β and γ subunits (I) in a total volume of 50 μl for 12 hours at 4°C before being incubated with retina sections. All other manipulations were at room temperature. Arrows represent the following: (A) the first appearance of α-transducin detected with antibody in 18-day embryo photoreceptors; (D) autofluorescent oil droplets (*od*) of cone photoreceptors; (E and F) immunostaining of rods (*r*) but not cones (*c*).

and sequenced in four laboratories; the nucleotide sequence reported by Lochrie *et al.* (*24*) differs in many respects from the sequence reported by Tanabe *et al.* (*25*), Medynski *et al.* (*26*) or Yatsunami and Khorana (*27*). The differences in nucleotide sequences of α-transducin cDNA's may be due to differences between bovine strains (*24*). Alternatively, the species of α-transducin cDNA described by Lochrie *et al.* may be expressed in cones, whereas the species cloned by the other groups may be expressed in rods (*28*). Further work is needed to determine whether different genes for α-transducin are expressed in rods and cones.

REFERENCES AND NOTES

1. E. A. Schwartz, *Annu. Rev. Neurosci.* **8**, 339 (1985).
2. B. K. K. Fung, J. B. Hurley, L. Stryer, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 152 (1981).
3. J. S. Pober and M. W. Bitensky, *Adv. Cyclic Nucleotide Res.* **11**, 265 (1979).
4. J. Nathans and D. S. Hogness, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 4851 (1984).
5. Unpublished results of J. Nathans and D. Hogness cited by L. Stryer, *Nature (London)* **312**, 498 (1984).
6. R. L. Hurwitz, A. H. Bunt-Milan, J. A. Beavo, *J. Biol. Chem.* **259**, 8612 (1984).
7. D. B. Farber and D. Bok, *J. Cell Biol.* **99** (part 2), 64a (1984).
8. D. B. Farber, D. W. Souza, D. G. Chase, R. N. Lolley, *Invest. Ophthalmol. Visual Sci.* **20**, 24 (1981).
9. S. J. Berger *et al.*, *J. Biol. Chem.* **255**, 3128 (1980).
10. R. A. Ortez, A. Tamayo, C. Johnson, H. G. Sperling, *J. Histochem. Cytochem.* **31**, 1305 (1983).
11. L. Haynes and K. W. Yau, *Nature (London)* **317**, 61 (1985).
12. W. H. Cobbs, A. E. Barkdoll III, E. N. Pugh, Jr., *ibid.*, p. 64.
13. D. Atwell, *ibid.*, p. 14.
14. D. R. Manning and A. G. Gilman, *J. Biol. Chem.* **258**, 7059 (1983).
15. P. Gierschik, J. Codina, C. Simons, L. Birnbaumer, A. Spiegel, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 727 (1985).
16. M. Pines, P. Gierschik, G. Milligan, W. Klee, A. Spiegel, *ibid.*, p. 4095.
17. J. B. Hurley, H. K. W. Fong, D. B. Teplow, W. J. Dreyer, M. I. Simon, *ibid.* **81**, 6948 (1984).
18. P. Witkovsky, *Vision Res.* **3**, 341 (1963).
19. V. B. Morris, *J. Comp. Neurol.* **140**, 359 (1970).
20. M. D. Olsen, *Anat. Rec.* **193**, 423 (1979).
21. K. Meller, *Anat. Embryol.* **169**, 141 (1984).
22. M. G. F. Fuortes and P. M. O'Bryan, in *Handbook of Sensory Physiology*, M. G. F. Fuortes, Ed. (Springer-Verlag, Berlin, 1972), vol. 7, part 2, p. 321.
23. D. A. Baylor, T. D. Lamb, K. W. Yau, *J. Physiol. (London)* **288**, 613 (1979).
24. M. A. Lochrie, J. B. Hurley, M. I. Simon, *Science* **228**, 96 (1985).
25. T. Tanabe *et al.*, *Nature (London)* **315**, 242 (1985).
26. D. C. Medynski *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 4311 (1985).
27. K. Yatsunami and H. G. Khorana, *ibid.*, p. 4316.
28. α-Transducin cDNA clones sequenced by Tanabe *et al.* (*25*), Medynski *et al.* (*26*), and Yatsunami and Khorana (*27*) were obtained with antibodies to bovine rod α-transducin, whereas Lochrie *et al.* (*24*) used oligodeoxynucleotide probes to detect α-transducin clones. Since antibodies can discriminate between α subunits of N_s, N_i, N_o, and transducin (*15*), it is likely that α-transducin clones sequenced by the three groups using the antibodies (*25, 26, 27*) corresponds to rod α-transducin.
29. G. B. Grunwald *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 4008 (1985).

26 August 1985; accepted 11 December 1985

Technical Comments

Carbon Dioxide Enhancement of Tree Growth at High Elevations

The apparent acceleration of tree growth by increased atmospheric carbon dioxide shown by LaMarche *et al.* (*1*) must be put in perspective lest it be argued that stimulation of growth of natural vegetation by an increase in CO_2 has been confirmed and that future research need only determine how best to take advantage of that fact.

Widths of annual rings of bristlecone and limber pine trees near timberline in central Nevada were 106 percent greater in the decade ending in 1983 than in the years 1850 to 1859 at one site and 73 percent greater at another (*1*). The estimated increase in atmospheric CO_2 concentration during the same period was 26 percent. Plant physiologists agree that even under favorable laboratory conditions the growth response of plants exposed to elevated CO_2 is no more than about half the corresponding increase in CO_2, whereas the increase in ring width is three to four times the CO_2 increment.

Tree condition may explain the discrepancy. The small annual rings—0.5 mm per year compared with the 4 to 8 mm per year typical of vigorous young forests—suggest that the sampled trees were fully mature, even senescent. Old trees have a high ratio of cambial and root respiration to photosynthesis, so that most photosynthate is used for maintenance. Even a slight increase in photosynthesis, whether from CO_2 increase or from another cause, could add disproportionately to the small surplus of photosynthate available to form new wood.

LaMarche *et al.* argue that effects of CO_2 increase on plant growth are greater at high elevation than near sea level. The partial pressure of CO_2 within photosynthesizing leaves is nearly independent of elevation. The partial pressure of atmospheric CO_2 is, however, appreciably lower at high altitudes, so the atmosphere-to-leaf gradient is smaller at high elevations than at low ones. A given percentage increase in CO_2 would therefore enhance this gradient more at high elevations. Several factors seemingly reduce the significance of this effect.

The diffusion coefficient of CO_2 is known experimentally to be inversely proportional to pressure. Gale (*2*), in a study cited by LaMarche *et al.*, showed that faster diffusion of CO_2 at high elevation partially compensates for the smaller atmosphere-to-leaf gradient. The degree of compensation depends on the ratio of boundary plus stomatal resistances, which are pressure (diffusion coefficient) dependent, to mesophyll (or carboxylation) resistance, which is not. The higher the ratio, the smaller the elevation effect (*2*). Increased mole fraction of CO_2 increases the ratio, since stomatal opening, and hence leaf conductance, is inversely correlated with atmospheric CO_2 concentration. This further diminishes the effect of elevation on sensitivity to CO_2 increase.

Reduction of photorespiration, one of the principal mechanisms by which increased atmospheric CO_2 enhances net photosynthesis (*3*), is unaffected by elevation. The bifunctional enzyme ribulosebisphosphate carboxylase catalyzes oxidation of newly formed carboxylate before it can enter subsequent steps of the photosynthetic cycle. The balance between carboxylation and oxygenation depends on the proportions of CO_2 and O_2 in the atmosphere (*4*), which at any given time are constant at all elevations of biological interest.

Quantitative assessment of the ecological and economic importance of a CO_2 effect on natural vegetation awaits analysis of growth response in representative forest stands in diverse climatic and soil conditions.

CHARLES F. COOPER
Department of Biology,
San Diego State University,
San Diego, CA 92182

REFERENCES AND NOTES

1. V. C. LaMarche, Jr., D. A. Graybill, H. C. Fritts, M. R. Rose, *Science* **225**, 1019 (1984).
2. J. Gale, *Ecology* **53**, 494 (1972).
3. E. R. Lemon, Ed., *CO_2 and Plants: The Response of Plants to Rising Levels of Atmospheric Carbon Dioxide* (Westview, Boulder, CO, 1983).
4. W. L. Ogren, *Annu. Rev. Plant Physiol.* **35**, 415 (1984).

11 December 1984; accepted 19 March 1985

LaMarche *et al.* (*1*) present tree ring data for trees growing at high altitude and suggest that apparent increases in growth from about 1850 to the present can be correlated with increasing concentrations of CO_2 in the atmosphere. They further argue that, at high altitudes, as a result of the lowered