tal cell numbers were substantially increased as well, indicating cell division (Table 1).

A direct effect of cholera toxin is indicated by the larger number of end buds in treated glands than in contralateral glands that received blank implants (Fig. 1 and Table 1). A slight systemic effect is also apparent if transplanted glands from untreated mice (mice without implants) are compared with glands from animals carrying a cholera toxin implant (Table 1). The specificity of the effect is indicated by the finding that the intact cholera toxin molecule had a substantially greater influence than its A or B subunits administered separately (Table 1).

Although systemically administered cholera toxin results in hyperplastic or dysplastic responses in skin (*20*), abnormalities were not observed in cholera toxin–treated senescent mammary tissue. After 10 days of cholera toxin stimulation the implant's effects were largely exhausted (*16*), the end buds had regressed, and there was no evidence of hyperplasia or other abnormal growth states.

The end buds formed in response to treatment were typically multilayered, with a distinct layer of undifferentiated cap cells (Fig. 2e), the putative stem cell population from which myoepithelial and perhaps other mammary cell types are derived (*21*). The reappearance of stem cells and the normal morphology of the end buds indicate that the tight coupling between cell proliferation and branching morphogenesis, characteristic of young tissue, remains intact.

In vitro, noncycling cells in certain lines of human fibroblasts have been stimulated to enter DNA synthesis in response to supplementation of the medium with cortisol (*22*). This response was possible, according to one interpretation, only because the cells tested had been propagated in medium that was hormonally suboptimal. Other reported cases of reinitiation of DNA synthesis in nuclei of senescent human fibroblasts involved fusion with HeLa or SV-40 transformed cells (*23*) or infection of senescent cells with DNA tumor viruses (*24*). Our results suggest that serially aged tissue can be restored, at least during treatment, to normal levels of growth by an agent that is not oncogenic and whose mode of action is expressed on well-studied metabolic pathways. Because we used whole glands we do not know whether the primary action of cholera toxin is on the epithelial or the stromal components.

Earlier investigations showed that growth-exhausted cells in mammary tissue passaged for long periods appear morphologically normal rather than exhibiting the generalized degenerative changes characteristic of senescent cells in culture. We have also reported that aged mammary ductal cells are capable of responding to the hormones of pregnancy by proliferating to the stage of alveolar differentiation, again indicating that major cell functions remain intact (*25*). The ability of cholera toxin to stimulate DNA synthesis in serially aged mammary cells further supports the interpretation that mitotic senescence is associated with age-related alterations in one or more regulatory pathways, which, in the case of mammary cells, may be regulated by cyclic AMP. The present results provide a temporarily "reversible" aging system that will be useful for the study of these interactions.

CHARLES W. DANIEL
GARY B. SILBERSTEIN
PHYLLIS STRICKLAND
Department of Biology, Thimann Laboratories, University of California, Santa Cruz 95064

References and Notes

1. L. Hayflick and P. S. Moorhead, *Exp. Cell Res.* **25**, 585 (1961).
2. L. Hayflick, *ibid.* **37**, 614 (1965).
3. C. W. Daniel, in *Handbook of the Biology of Aging*, C. Finch and L. Hayflick, Eds. (Reinhold, New York, 1977), pp. 122–154.
4. C. W. Daniel, K. B. DeOme, J. T. Young, P. B. Blair, L. J. Faulkin, *Proc. Natl. Acad. Sci. U.S.A.* **61**, 52 (1968).
5. C. W. Daniel, J. M. Shannon, G. R. Cunha, *Mech. Ageing Dev.*, in press.
6. C. W. Daniel, B. D. Aidells, D. Medina, L. J. Faulkin, Jr., *Proc. Fed. Am. Soc. Exp. Biol.* **34**, 64 (1975).
7. J. Moss and M. Vaughan, *Annu. Rev. Biochem.* **48**, 581 (1979).
8. M. Green, *Cell* **16**, 801 (1978).
9. C. L. Marcelo, *Exp. Cell Res.* **120**, 201 (1979).
10. A. Guidotti, B. Weiss, E. Costa, *Mol. Pharmacol.* **8**, 521 (1972).
11. U. Armato, E. Draghi, P. G. Andrein, *Exp. Cell Res.* **105**, 337 (1977).
12. M. R. Stampfer, *In Vitro* **18**, 531 (1982).
13. J. Yang, R. Guzman, W. Imagawa, K. McCormick, S. Nandi, *Endocrinology* **107**, 35 (1980).
14. J. A. Rillema, *Proc. Soc. Exp. Biol. Med.* **151**, 748 (1976).
15. W. Kung, E. Bechtel, E. Geyer, *FEBS Lett.* **82**, 102 (1977).
16. G. B. Silberstein, P. Strickland, V. Trumpbour, S. Coleman, C. W. Daniel, *Proc. Natl. Acad. Sci. U.S.A.*, in press.
17. G. B. Silberstein and C. W. Daniel, *Dev. Biol.* **93**, 272 (1982).
18. W. D. Rhine, D. S. T. Hseih, R. L. Langer, *J. Pharmacol. Sci.* **69**, 265 (1980).
19. K. B. DeOme, L. J. Faulkin, H. A. Bern, P. B. Blair, *Cancer Res.* **19**, 515 (1959).
20. F. V. Schaefer, R. P. Custer, S. Sorof, *Nature (London)* **286**, 807 (1980).
21. J. M. Williams and C. W. Daniel, *Dev. Biol.* **97**, 274 (1983).
22. P. D. Phillips, K. Woolwich, V. J. Cristofalo, *Mech. Ageing Dev.* **20**, 271 (1982).
23. T. H. Norwood, W. R. Pendergrass, G. M. Martin, *J. Cell Biol.* **64**, 551 (1975).
24. T. Ide, Y. Tsuji, S. Ishibashi, Y. Mitsui, *Exp. Cell Res.* **143**, 343 (1983).
25. C. W. Daniel, L. J. T. Young, D. Medina, K. B. DeOme, *Exp. Gerontol.* **6**, 95 (1971).
26. We thank L. Wilson for technical assistance and V. Trumpbour and S. Coleman for providing helpful information and suggestions. Supported by PHS grant 01050 from the National Institute on Aging.

6 February 1984; accepted 5 April 1984

Dietary Restriction Retards Age-Related Loss of Gamma Crystallins in the Mouse Lens

Abstract. *The soluble crystallins in lenses from diet-restricted and control mice of diverse ages (2, 11, or 30 months) were studied by high-performance liquid chromatography and sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Results obtained with both methods suggest that dietary restriction decelerates age-related loss of soluble gamma crystallins.*

Dietary restriction, when started early in life and continued until death, increases average and maximal survival times of mice (*1*) and rats (*2*). It extends maximal survival times more reliably than other antiaging strategies tested in rodents (*3*) and is the only procedure yet tested in homeotherms that slows age-related increases in mortality rates (*4*). Longer life-spans for rodents on restricted diets are associated with a lower incidence and later onset of several late-life diseases (*5*). Rodents on diet restriction show more youthful physiologic (*6*) and immunologic (*7*) responses than do age-matched controls on unrestricted diets. The antiaging action of dietary restriction appears to depend on restriction of calories (a 25 to 50 percent decrease in most studies) without a decrease in essential nutrients (undernutrition without malnutrition).

For many years the eye lens has been a model for studying age-related biochemical changes in structural proteins and enzymes (*8, 9*). The lens of the vertebrate eye is composed primarily of concentric layers of long hexagonal units called lens fiber cells. These fiber cells differentiate from an epithelial monolayer that covers the anterior surface of the lens and terminates at the equator. During the process of differentiation (which occurs throughout life), the equatorial epithelial cells elongate and follow a curved course that extends from the anterior to the posterior pole of the lens. New lens fibers are deposited over the older ones, the latter being compressed toward the interior of the region known

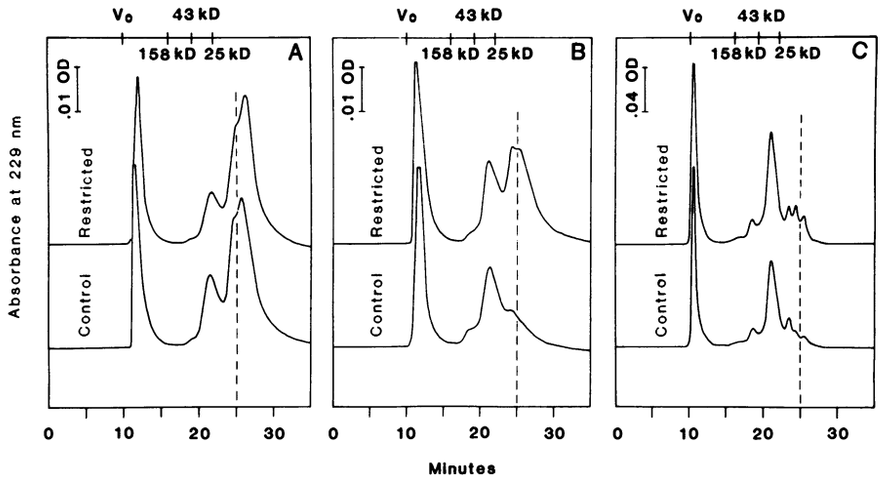

Fig. 1. HPLC elution profiles of soluble proteins from lenses of diet-restricted and control mice aged (A) 2 months, (B) 11 months, and (C) 30 months. Gel filtration of individual lens supernatants was performed essentially as described by Horwitz and his co-workers (*17*). Briefly, two columns (TSK-3000, 7.5 by 300 mm, Toyosoda, Japan) and a guard column (TSK-3000 SW, 7.5 by 100 mm) were connected in series to an HPLC system (Beckman, model 324M). The proteins were monitored with a detector (Beckman, model 160) at 229 nm. Samples were eluted with a buffer containing 0.05*M* sodium phosphate and 0.1*M* Na_2SO_4, *p*H 7, at a flow rate of 1.0 ml/min. Each profile is a representative of an individual lens (n = 4, by age and by diet). Within each age and diet group the chromatograms were highly reproducible and superimposable under identical conditions. Abbreviations: V_0, void volume; *OD*, optical density.

as the nucleus. As a result of this constant process of differentiation in the absence of cell shedding, the lens gradually increases in size with age. The fiber cells occupying the superficial layers (cortical fibers) begin to lose their mitochondria, nuclei, and cytoplasmic membranes shortly after elongation starts but retain their polyribosomes for a while (*10*). During this time they synthesize large amounts of structural proteins called crystallins. The oldest fiber cells of the lens become compacted into the lens center and, having lost all their organelles, are incapable of protein synthesis (*11*). The changes in distribution and composition of the water-soluble lens crystallins in rodents during aging has been studied by several investigators (*8, 9, 12*). A consistent finding is a marked decrease in the amount of soluble gamma crystallin with aging. In man, loss of gamma crystallin also occurs with age and is associated with cataract development (*9, 13*).

We now report the effects of dietary restriction on age-related distribution of gamma crystallin in the mouse lens. A long-lived F_1 hybrid mouse strain was investigated. Females of the C3H.SW/Sn inbred strain were mated with males of the C57BL/10.RIII/Sn inbred strain in our animal facilities. Female hybrid progeny were weaned at 21 to 24 days of age, individually caged, and randomly assigned to one of two diet groups. The mice were fed semipurified diets providing either 85 kcal per week (controls) or 50 kcal per week (restricted diet group). Diet composition, feeding strategy, and body weight gain were as previously reported (*14*). Mice in the restricted diet group ate limited amounts of a diet enriched in protein (casein), vitamins, and salt content, so that their weekly intakes of these nutrients approximated those of controls fed nonenriched diets. Weekly intakes of carbohydrate (sucrose and cornstarch) and fat (corn oil) were less for the mice on the restricted diet than for the controls. Adult body weights averaged about 35 g for the controls and about 20 g for the restricted diet group. We have observed (*15*) that mice of this F_1 hybrid strain show a mean life-span of about 33 months for controls (n = 57) versus about 43 months for mice on the restricted diet (n = 71).

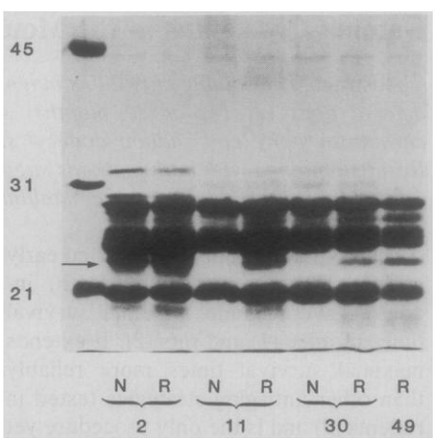

Fig. 2. Results of SDS-PAGE of soluble proteins from lenses of diet-restricted (*R*) and normally fed control (*N*) mice aged 2, 11, and 30 months and from diet-restricted (R) mice aged 49 months. Samples (5 μl) were resolved on 10 percent polyacrylamide gels and stained with Coomassie brilliant blue. Molecular weight standards were ovalbumin (45 kD), carbonic anhydrase (31 kD), and soybean trypsin inhibitor (21 kD). To facilitate the identification of the 23-kD band (arrow), we transferred the SDS gels to nitrocellulose and stained them, first with rabbit antibodies to gamma crystallin and then with ^{125}I-labeled protein A. The autoradiogram confirmed the identity of the 23-kD band as a gamma crystallin.

Freshly dissected lenses were individually weighed, homogenized in Millipore-filtered buffer containing 0.05*M* sodium phosphate and 0.1*M* Na_2SO_4, *p*H 7 (Wheaton 0.1-ml tissue grinder), and centrifuged (Eppendorf Micro Centrifuge 5414) for 30 minutes at 4°C. The supernatant fractions were reserved for protein determination (*16*) and for analysis by high-performance liquid chromatography (HPLC) (*17*) and one-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (*18*).

Supernatants prepared from individual lenses of restricted-diet and control mice of diverse ages (2, 11, and 30 months old; four mice of each age in each diet group) were subjected to gel filtration chromatography with an HPLC system. The HPLC elution profiles of the lenses from 2-month-old mice were almost identical (Fig. 1A) in the restricted-diet and control groups. The first peak, representing material eluting at about 11 minutes, contained the high molecular weight proteins and alpha crystallins (Fig. 1, A to C). The second peak at about 21 minutes contained the beta crystallins. The third peak at about 26 minutes contained the gamma crystallins and the low molecular weight fraction. The elution profiles of lenses from 11-month-old restricted and control mice show dramatic age and diet-related differences (Fig. 1B). In four comparisons, we consistently found that the amount of gamma crystallins in the lenses from 11-month-old mice on restricted diets was significantly greater than that in lenses from age-matched controls. This effect of long-term diet restriction on the loss of gamma crystallins also was apparent at 30 months (Fig. 1C). To our knowledge, these findings represent the first reported deceleration of age-related loss of gamma crystallins.

To further characterize these differences, we analyzed equal amounts of protein from the same lens samples by SDS-PAGE using the method of Laemmli (*18*). Results from each analysis (Fig. 2) confirmed the impressions gained from chromatography data. The banding patterns of the lenses from the 2-month-old control mice and those on restricted diets were essentially identi-

cal. A major band at about 23 kD, which was present at 2 months, was retained by lenses from mice on the restriced diet at 11, 30, and even at 49 months, but was missing in the age-matched controls. There were no controls at 49 months because mice on the control diet do not live that long. Additional experiments were performed on lens supernatants from age-matched mice on restricted and control diets at ages 21, 24, 25, and 28 months and on the lenses from mice on restricted diets at ages 41, 44, and 49 months. In all cases, the HPLC profiles or the SDS-PAGE results, or both, showed that lenses from animals on the restricted diet retained larger quantities of soluble gamma crystallins (data not shown).

In rodents and many other species, there is a progressive decrease with age in the concentration of the soluble structural protein, gamma crystallin (*8, 9, 12*). The precise mechanism for this decline remains to be determined. Using Raman spectroscopy, Kuck *et al.* (*19*) monitored the total sulfydryl concentration in the intact lenses of rats and mice. They observed a rapid loss with age of SH groups concomitant with an increased insolubilization of gamma crystallins. Free SH groups are also lost with aging in other proteins, including nonhistone chromatin (*20*) and serum albumin (*21*). It would be interesting to determine whether diet restriction influences the free SH concentration in the lens. Such an effect might help to clarify the role of the high concentrations of free SH groups found in the lens. Our results indicate that animals on dietary restrictions can provide a useful experimental model for the study of aging processes in the eye.

P. J. LEVEILLE

Mental Retardation Research Center, School of Medicine, University of California, Los Angeles 90024

R. WEINDRUCH

R. L. WALFORD

Department of Pathology, School of Medicine, University of California, Los Angeles

D. BOK

J. HORWITZ*

Jules Stein Eye Institute, School of Medicine, University of California, Los Angeles

References and Notes

1. M. J. Tucker, *Int. J. Cancer* **23**, 803 (1979); K. E. Cheney, R. K. Liu, G. S. Smith, P. J. Meredith, M. R. Mickey, R. L. Walford, *J. Gerontol.* **38**, 420 (1983).
2. C. M. McCay, M. F. Crowell, L. A. Maynard, *J. Nutr.* **10**, 63 (1935); M. H. Ross, *ibid.* **75**, 197 (1961); B. P. Yu, E. J. Masoro, I. Murata, H. A. Bertrand, F. T. Lynd, *J. Gerontol.* **37**, 130 (1982).
3. R. L. Walford, *The Immunologic Theory of Aging* (Munksgaard, Copenhagen, 1969), pp. 104–111; M. H. Ross, in *The Biology of Aging*, J. A. Behnke, C. E. Finch, G. B. Moment, Eds. (Plenum, New York, 1978), p. 173; R. G. Cutler, in *Aging: Biology and Behavior*, J. L. McGaugh and S. B. Kiesler, Eds. (Academic Press, New York, 1981), p. 31.
4. G. A. Sacher, in *Handbook of the Biology of Aging*, C. E. Finch and L. Hayflick, Eds. (Van Nostrand Reinhold, New York, 1977), p. 582.
5. A. Tannenbaum, *Cancer Res.* **2**, 460 (1942); B. N. Berg and H. S. Simms, *J. Nutr.* **71**, 255 (1960); M. H. Ross and G. Bras, *ibid.* **87**, 245 (1965); B. P. Yu *et al.*, *J. Gerontol.* **37**, 130 (1982).
6. E. J. Masoro, B. P. Yu, H. A. Bertrand, F. T. Lynd, *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **39**, 3178 (1980); H. A. Bertrand, in *Review of Biological Research in Aging*, M. Rothstein, Ed. (Liss, New York, 1983), p. 359.
7. R. H. Weindruch, J. A. Kristie, K. E. Cheney, R. L. Walford, *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **38**, 2007 (1979); R. H. Weindruch, S. R. S. Gottesman, R. L. Walford, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 898 (1982).
8. S. Lerman and S. Zigman, *Acta Ophthalmol.* **45**, 193 (1967); J. Bours, *Comp. Biochem. Physiol. B* **65**, 215 (1980); J. Bours and O. Hockwin, *Ophthalmic Res.* **15**, 234 (1983); J. G. Bindels, J. Bours, H. J. Hoenders, *Mech. Ageing Dev.* **21**, 1 (1983).
9. H. Bloemendal, Ed. *Molecular and Cellular Biology of the Eye Lens* (Wiley, New York, 1981).
10. E. L. Benedetti, I. Dunia, C. J. Bentzel, A. J. M. Vermorken, M. Kibbelaar, H. Bloemendal, *Biochim. Biophys. Acta* **457**, 353 (1976).
11. R. W. Young and H. W. Fulhorst, *Invest. Ophthalmol.* **5**, 288 (1966).
12. P. Russell, S. G. Smith, D. A. Carper, J. H. Kinoshita, *Exp. Eye Res.* **29**, 245 (1979); T. Uchiumi, S. Kimura, K. Ogata, *ibid.* **36**, 125 (1983).
13. H. Maisel and M. Goodman, *Invest. Ophthalmol.* **4**, 129 (1965).
14. R. H. Weindruch, J. A. Kristie, F. Naeim, G. B. Mullen, R. L. Walford, *Exp. Gerontol.* **17**, 49 (1982).
15. R. Weindruch, S. Fligiel, D. Guthrie, R. L. Walford, unpublished data.
16. O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951); M. M. Bradford, *Anal. Biochem.* **72**, 248 (1976).
17. J. Horwitz, R. Neuhaus, J. Dockstader, *Invest. Ophthalmol. Vis. Sci.* **21**, 616 (1981); J. Horwitz, L. L. Ding, C. C. Cheung, *Lens Res.* **1**, 159 (1983).
18. U. V. Laemmli, *Nature (London)* **227**, 680 (1970).
19. J. F. R. Kuck, N. T. Yu, C. C. Askren, *Exp. Eye Res.* **34**, 23 (1982).
20. S. Tas, C. F. Tam, R. L. Walford, *Mech. Ageing Dev.* **12**, 65 (1980).
21. S. Leto, M. J. Yiengst, C. H. Barrows, Jr., *J. Gerontol.* **25**, 4 (1970).
22. We thank S. J. Zigler, NIH, for antibodies to gamma crystallin, and L.-L. Ding and G. McFeeters for technical assistance. This study was supported by PHS grants EY00444, EY3897, and AG00424.
* To whom correspondence should be addressed.

3 February 1984; accepted 2 May 1984

Enzymatic Catalysis in Organic Media at 100°C

Abstract. *Porcine pancreatic lipase catalyzes the transesterification reaction between tributyrin and various primary and secondary alcohols in a 99 percent organic medium. Upon further dehydration, the enzyme becomes extremely thermostable. Not only can the dry lipase withstand heating at 100°C for many hours, but it exhibits a high catalytic activity at that temperature. Reduction in water content also alters the substrate specificity of the lipase: in contrast to its wet counterpart, the dry enzyme does not react with bulky tertiary alcohols.*

Water has a dual effect in enzymatic systems: it is essential for acquisition and maintenance of the enzymes' catalytically active conformation (*1*), and it is required for most enzyme inactivation processes, in particular those of thermal inactivation (*2*). Several enzymes have been shown to function in lipophilic organic media in the presence of a few percent of water (*3*). We now report that, upon further dehydration, enzymes exhibit some new catalytic properties.

Porcine pancreatic lipase (*4*), a well-characterized enzyme (*5*), was used as a model in most of our work. We investigated the lipase-catalyzed transesterification reaction between tributyrin (tributyryl glycerol) and various alcohols and found that the reactions yielded a monoester of butyric acid and dibutyrin. The substrate mixture (usually a 2*M* solution of an alcohol in tributyrin) also served as the reaction medium. A powder of the enzyme was added to the reaction mixture, and the suspension was shaken vigorously at 20°C; samples were periodically withdrawn and analyzed by gas chromatography (*6*). The water concentration, measured by the optimized method of Fischer (*7*), was controlled (i) in the solvent by passing it through a molecular sieve column and subsequently adding a known amount of water and (ii) on the enzyme by evacuating the powder under vacuum.

The rate of the lipase-catalyzed reaction between tributyrin and heptanol was studied as a function of the concentration of water in the liquid phase in the range from 0 to 1.1 percent (Fig. 1) (*8*). The enzyme remained catalytically active at concentrations of water as low as 0.015 percent. Even when the amount of water adsorbed on the enzyme (0.48 percent) was included, the water content of the system sufficient for the lipase to act as a catalyst was still less than 0.02 percent. Virtually the same profile was observed for the enzyme preparation that contained 3.6 percent water (the "wet" lipase). We measured the water content of the lipase powders after they were incubated in the transesterification reaction mixture and found that the "dry" lipase (0.48 percent initial water content), when placed in the reaction mixture with 0.8 percent water, adsorbed water from the solvent; after 3 hours of incubation the powder contained 3.6 percent water. The wet lipase