

the traumatized appendage had become severely atrophied and 80 to 90 percent of it was necrotic, so it is likely that the innervation of the area was drastically affected (11).

In conclusion, hypovolemia produced by ultrafiltration of the blood is a sufficient stimulus for thirst in sheep, and crushing the left atrial appendage eliminates this behavioral response. These findings add to the advances made during the past decade in elucidating the physiological bases of thirst—especially in the identification of relevant stimuli and the location of receptors on which they act (4, 15, 18). To date, however, destruction of such receptor sites in the central nervous system has invariably disrupted the drinking response to more than one stimulus (19). Our results may constitute the first example of neural damage that specifically eliminates the drinking response to one stimulus for thirst.

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6. Although rats were used as subjects in most previous work on hypovolemic thirst, it is more convenient to use a larger animal to assess the role of vascular stretch receptors. The size and docility of sheep, as well as the clear indication that they increase water intake in response to the loss of extracellular fluid [S. F. Abraham, J. P. Coghlan, D. A. Denton, J. G. McDougall, D. R. Mouw, B. A. Scoggins, *Q. J. Exp. Physiol.* **61**, 185 (1976); (8)], made them admirably suited for this study.
7. We used the Gambro lundia plate (surface area, 1.36 m²; membrane thickness, 11.5 μm).
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9. All the sheep had been conditioned to a feeding schedule (3 p.m. to 5 p.m.) and a schedule of water availability (3 p.m. to 9 a.m. the next morning). Increases in water intake after ultrafiltration were determined by subtracting the average amount of water consumed by each sheep during the baseline period (5 to 6 days before a filtration session) from that consumed after the establishment of hypovolemia.
10. Changes in plasma volume were calculated as $(PP_1 - PP_2)/PP_2 \times 100$ [E. M. Stricker, *Physiol. Behav.* **3**, 379 (1968)], where PP_1 is the basal protein concentration and PP_2 is the protein concentration at the end of the filtration period.
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12. Atrial damage led to chronic tachycardia, as

noted previously (11). Nevertheless, basal water intakes measured in seven sheep before and after the left atrial appendage was crushed were not significantly different; before surgery, the mean intakes were 1638 ± 115 and 2312 ± 230 ml during 2 and 18 hours of water availability, respectively; after surgery they were 1499 ± 128 and 2253 ± 164 ml.

13. Operated sheep lost 1.5 kg in body weight despite normal daily intakes of food and water. Some portion of this undoubtedly represents extracellular fluid lost during surgery and not subsequently replaced (the sheep were maintained on a diet of alfalfa pellets and tap water, which provided only 8 mEq of sodium per day).
14. Furosemide was administered intravenously in isotonic saline (0.21 ml/min for 4 hours). The diuretic was given on two consecutive days at doses of 250 and 125 mg per sheep. Urine collected through a bladder catheter contained 125 ± 8 mEq of sodium.
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17. Due to their maintenance on a low-sodium diet (13), neither group excreted much of the salt

load. The significant difference in their intakes reflects the fact that the unoperated sheep did not drink enough water to dilute the remaining salt load to isotonicity, whereas the sheep with crushed atrial appendages did. It is possible that the surgery abolished tonic inhibitory impulses from low-pressure volume receptors and thereby lowered the threshold for osmoregulatory drinking [S. Kozlowski and E. Szczepanska-Sadowska, in *Control Mechanisms of Drinking*, G. Peters, J. T. Fitzsimons, L. Peters-Haefeli, Eds. (Springer-Verlag, New York, 1975), pp. 25–35].

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20. Preliminary versions of this report were presented at the 9th Annual Meeting of the Society for Neuroscience, Atlanta, 2 to 6 November 1979, and the 7th International Conference on the Physiology of Food and Fluid Intake, Warsaw, 7 to 10 July 1980. Correspondence should be addressed to M.B.Z.

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Nonenzymatic Browning in vivo: Possible Process for Aging of Long-Lived Proteins

Abstract. *The incubation of lens proteins with reducing sugars leads to the formation of fluorescent yellow pigments and cross-links similar to those reported in aging and cataractous human lenses. Called nonenzymatic browning or the Maillard reaction, this aging process also occurs in stored foods. Reducing sugars condense with the free amino group of proteins, then rearrange and dehydrate to form unsaturated pigments and cross-linked products. Although most proteins in living systems turn over with sufficient rapidity to avoid nonenzymatic browning, some, such as lens crystallins and skin collagen, are exceptionally long-lived and may be vulnerable.*

The initial stage of the Maillard reaction, nonenzymatic glycosylation, has been shown to occur in vivo (1). Glucose or glucose-6-phosphate reacts with the terminal amino group or the ε-amino group of lysine residues in proteins. The condensation product, an aldiosylamine, undergoes an Amadori rearrangement to

form a 1-deoxyfructosyl adduct. Amadori products have been detected in hemoglobin (2), erythrocyte membrane protein (3), lens crystallins (4), collagen (5), albumin, and serum proteins (6). In the later stages of the Maillard reaction, during nonenzymatic browning, the Amadori product undergoes multiple dehydrations to form yellow-brown fluorescent products and protein cross-links that decrease the protein solubility (1).

Similar changes have been observed in the aging and cataractous human lens. These include protein aggregation (7), decreased protein solubility (8), oxidation of sulfhydryl groups (9), production of nondisulfide covalent cross-links be-

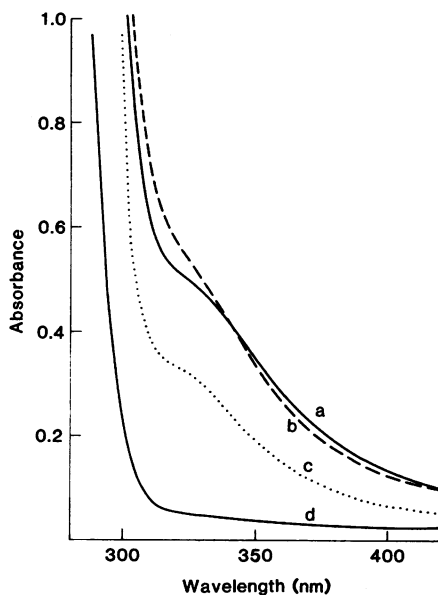


Fig. 1. Absorption spectra of human and bovine lens protein digests, as recorded with a Beckman Acta CIII spectrophotometer. The concentration in all samples was 8 mg/ml in 50 mM ammonium bicarbonate buffer (pH 8.75). Spectra: a, cataractous human lens proteins; b, bovine lens proteins incubated with 5 mM glucose-6-phosphate; c, bovine lens proteins incubated with 5 mM glucose; and d, control lens (incubated without hexose). The spectrum of the digests from young noncataractous human lenses was identical with spectrum d.

tween proteins (10, 11), increased pigmentation in the lens nucleus (10, 12), and a dramatic increase in protein-bound blue fluorescence not attributable to tryptophan (13). Although a photooxidative effect has been implicated as a possible mechanism for protein cross-linking and pigmentation in the human lens (12, 14-16), the nature of the main protein-bound fluorophore and chromophore has not been elucidated.

The discovery of Amadori products with lysine residues in lens crystallins offers a new insight into the basis for the pigmentation and cross-linking based upon the nonenzymatic browning reaction with lysine residues. To study the effects of the Maillard reaction on lens proteins, we incubated sterile solutions of bovine cortical lens proteins (30 mg/ml) for 10 months at 37°C in 50 mM potassium phosphate buffer (pH 7.4) with 5 mM glucose or glucose-6-phosphate. The deaerated solutions were incubated in the dark in sealed tubes to prevent photooxidation. The solutions were subsequently dialyzed against 7M urea in 0.1M tris-HCl (pH 8.6) containing 50 mM dithioerythritol, and alkylated with iodoacetate. The alkylated proteins were dialyzed against 0.2M ammonium bicarbonate and digested four times with 1 percent (by weight) Pronase P (Sigma, type 6). The same procedure was used to prepare a protein digest of human lens proteins. Thirteen cataractous lenses [ages 55 to 72 years, stages 3 and 4 in Pirie's classification (10)] were decapsulated and stirred overnight at 4°C in 13 ml of deaerated 0.1M tris-HCl (pH 8.6). The homogenate was centrifuged at 20,000g for 20 minutes. The precipitated yellow insoluble fraction was reduced with dithioerythritol and alkylated as described above. The alkylated proteins were solubilized by digestion with Pronase.

The ultraviolet absorption and the fluorescence properties of the protein digest from browning products obtained in vitro were investigated and compared with those of digests of cataractous lenses. The absorption spectra were recorded from 300 to 400 nm (Fig. 1). A yellow pigment was formed when bovine lens proteins were incubated with glucose and glucose-6-phosphate. A similar absorption spectrum, with a shoulder at 330 nm, was detected in the protein digest of human cataracts. This similarity was more apparent when the fluorescence excitation maxima were compared. In addition to the excitation maximum with tryptophan, three new maxima at 360, 400, and 470 nm were detected in solutions of crystallins in-

culated with hexoses (Fig. 2A). These maxima closely resemble those observed for the protein digest of cataractous lenses (Fig. 2B). No similar excitation maximum was found for bovine lens crystallins incubated without sugar or for protein digests from 20- to 24-year-old normal human lenses prepared as described above. The emission maximum of the major fluorescent chromophore in the digest of cataractous lens proteins was detected at 430 nm when excited at 360 nm. Digests of bovine crystallins that

had been incubated with glucose and glucose-6-phosphate had emission maxima at 425 and 430 nm, respectively, when excited at 360 nm.

We also investigated whether nonenzymatic browning could account for the covalent nondisulfide cross-links observed in proteins of senile and cataractous lenses. The solutions of bovine crystallins incubated with hexoses were fractionated by gel filtration in urea on Sephadex G-200 in the presence of dithioerythritol. Nondisulfide cross-linked aggregates with a molecular weight greater than 200,000 were detected in crystallins incubated with glucose-6-phosphate (17 percent of total proteins) and glucose (5 percent of total proteins), but not in the control, which had been incubated without hexose. The increased amount of cross-linked proteins in samples incubated with glucose-6-phosphate is consistent with the greater browning reaction rate that generally occurs with phosphorylated reducing sugars (17).

Several lines of evidence support the possibility that nonenzymatic browning may be involved in aging and cataractogenesis. First, A. Pirie observed a 26 percent decrease in lysine residues and a 46 percent increase in tyrosine in hydrolysates of cataractous proteins when compared with native crystallins (1). When we analyzed an acid hydrolysate of α -tert-butylloxycarbonyllysine that had been incubated with radioactively labeled glucose, we observed a radioactive and ninhydrin-positive peak coeluting with tyrosine (18). This suggests that the reported increase in tyrosine residues may be due in part to modified lysine residues. Second, Amadori products of lysine residues are present in lens crystallins (4) and accumulate in the aging lens (19). Finally, both the findings of Mohammad *et al.* (20) on the browning of albumin with glucose and our results suggest that the initially formed Amadori product is able to rearrange at physiological pH and temperature to form a more stable pigmented protein adduct. The lens may be particularly vulnerable, since it contains a high percentage of crystallins synthesized during fetal life and not turned over (21). Although the structures of the pigments and cross-links have not yet been identified, it is likely that osulose, *cis*-osulose-3-ene, and 5-hydroxymethylfurfuraldehyde are involved in the browning process (1).

Nonenzymatic browning may be an important factor in the aging of tissues that are insulin-independent and thus exposed to high glucose concentrations, such as the lens, the basement membrane of arteries, the nerves, and the in-

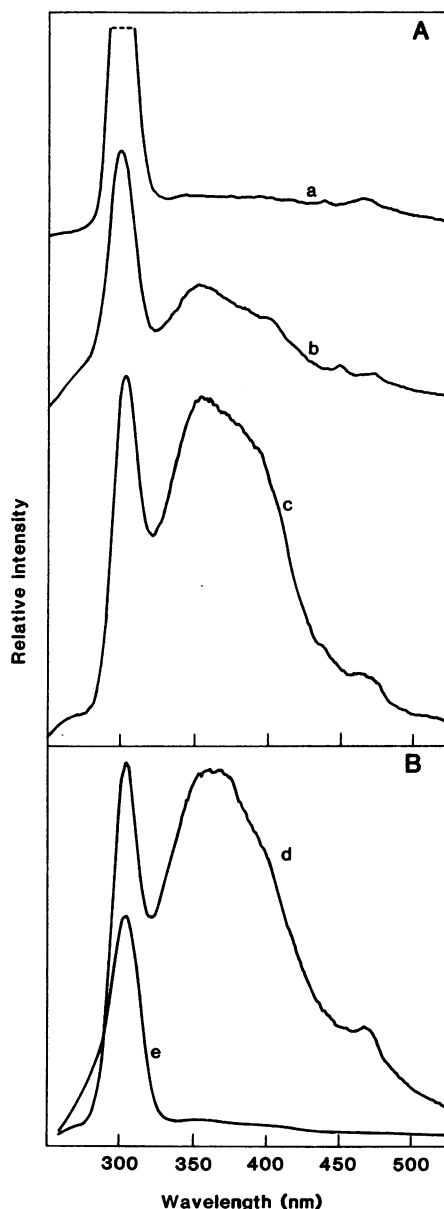


Fig. 2. Excitation spectra (uncorrected) of human and bovine lens protein digests, as recorded with a Perkin-Elmer model 204 fluorescence spectrophotometer. The concentrations are the same as in Fig. 1; the sensitivity for the spectra in (B) is four times less. (A) Bovine lens crystallins incubated without sugar (a), with 5 mM glucose (b), and with 5 mM glucose-6-phosphate (c). (B) Human lens proteins from cataractous (d) and young normal (e) lenses.

terstitial tissue of the skin. In diabetes, the Maillard reaction may accelerate aging in these tissues and contribute to the earlier onset of cataracts and atherosclerosis.

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Opiate Antagonist Improves Neurologic Recovery After Spinal Injury

Abstract. *The opiate antagonist naloxone has been used to treat cats subjected to cervical spinal trauma. In contrast to saline-treated controls, naloxone treatment significantly improved the hypotension observed after cervical spinal injury. More critically, naloxone therapy significantly improved neurologic recovery. These findings implicate endorphins in the pathophysiology of spinal cord injury and indicate that narcotic antagonists may have a therapeutic role in this condition.*

Traumatic injuries to the spinal cord may cause neurologic impairment in two ways—by directly interrupting neuronal pathways and by initiating a series of pathophysiologic changes that lead to progressive ischemic damage to the spinal cord (1). There is experimental evidence that these ischemic changes are potentially reversible and result at least in part from a reduction in spinal cord blood flow (2). Under normal circumstances, the spinal cord, like the brain, can maintain relatively constant blood flow over a wide range of blood pressures; such autoregulation is impaired after injury (3). When this occurs, perfusion of the cord becomes more directly dependent on systemic blood pressure. Since significant hypotension often accompanies injuries of the cervical or upper thoracic spinal cord (4), this combined loss of autoregulation and de-

creased systemic blood pressure may potentiate ischemic changes observed after trauma to these regions. We have recently demonstrated that the opiate antagonist naloxone reverses the hypoten-

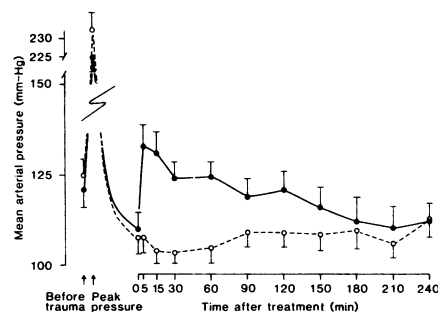


Fig. 1 Effects of naloxone ($N = 9$, filled circles) or saline ($N = 13$, open circles) treatment on mean arterial pressure after 500 g-cm trauma to the cervical spinal cord. Points represent averaged values \pm standard errors of the mean.

sion caused by transection of the cervical spinal cord, thereby implicating endorphins in the pathophysiology of spinal shock (5). If endorphin activation also contributes to the hypotension caused by cervical spinal injury, naloxone treatment should increase both systemic blood pressure and local spinal cord blood flow, thus limiting ischemic damage and improving neurologic function. The purpose of our studies, therefore, was to investigate the effects of parenterally administered naloxone on blood pressure and neurologic recovery after low cervical spinal injury.

Adult cats (2 to 3 kg) were anesthetized with intravenous pentobarbital (30 mg per kilogram of body weight), paralyzed with gallamine triethiodide, and placed on a ventilator. Blood pressure was continuously recorded on a Dynograph (Beckman) through the use of a femoral artery catheter connected to a pressure transducer (Statham). A femoral venous catheter permitted intravenous drug administration. A laminectomy was performed to expose spinal segments C_6 to T_1 ; with the dura intact, the C_7 spinal segment was traumatized by dropping a 20-g lead weight a distance of 25 cm (500 g-cm force) onto a 10-mm² plastic impact plate which had been contoured to match the curve of the spinal cord. This model, originally described by Allen (6), has been extensively used in studies of spinal cord injury (7). Pilot studies indicated that these injury variables would produce a moderately severe, reproducible spastic quadriplegia in untreated animals. Forty-five minutes after injury, animals were treated intravenously with equal volumes of either naloxone hydrochloride ($N = 9$, Endo Laboratories) or saline ($N = 13$). Blood pressure was recorded and drugs were administered continuously over a 4-hour treatment period with an infusion pump (Harvard, model 975); the naloxone dosage (a 2-mg bolus followed by 2 mg per kilogram per hour) was established from previous experiments (8, 9). Blood pressure was recorded during the 4 hours of treatment, after which the catheters were removed. The laminectomy site was closed in layers and the animal was allowed to recover in its home cage. Neurologic function was evaluated at 24 hours, 1, 2, and 3 weeks by two neurologists who were unaware of treatment or blood pressure findings. Neurologic status was rated according to an established five-point scale based primarily on motor function (10). Forelimb and hindlimb scores were determined separately as follows: 0, absence of voluntary movement; 1, minimal voluntary movement;