Abnormal Redox Status Without Increased Lipid Peroxidation in Sugar Cataract

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In conflict with a previous report, we find that phenolic inhibitors of lipid peroxidation (butylated hydroxytoluene [BHT] and butylated hydroxyanisole [BHA]) do not have significant inhibitory effect on galactosemic cataract formation. This is consistent with the lack of enhancement of stable products of lipid peroxidation (measured by the thiobarbituric acid assay) in the lenses of galactosemic rats. This does not imply that oxidative stress plays no role in galactosemic cataract formation (indeed, we find that galactosemic lens homogenates contain increased amounts of an Fe^{2+} oxidant, possibly a peroxide), but rather that BHT- and BHA-inhibitable lipid peroxidation specifically has no role to play. In instances where drugs appear to inhibit galactosemic cataract formation, other effects caused by the drugs, e.g., inhibition of feeding or induction of general detoxification pathways, must be considered. *Diabetes* 39:1347–52, 1990

The suggestion that oxidative stress contributes to galactosemic and diabetic cataract formation is based on the observations that *1*) polyol accumulation alone cannot account for the sequence of biochemical events culminating in lens opacification, *2*) various antioxidants have been suggested to protect against lens damage in these models, and *3*) the changes that occur in the lens during diabetes and galactosemia (e.g., loss of tryptophan fluorescence) are fully consistent with oxidative stress from some source (1). Three processes by which the normal redox status of the lens could be disrupted in galactosemia and diabetes have been proposed: *1*) monosaccharide autoxidation, which causes NAD(P)H oxidation, lipid peroxidation, and protein damage in vitro (2,3); *2*) competition between aldose reductase and glutathione reductase for NADPH, which could lead to a lowering of lenticular antioxidant defenses (4); and *3*) autoxidation of monosaccharide-protein (glycation) adducts, which occurs in the lens and would lead to free radical and hydrogen peroxide (H_2O_2) production (5).

Current emphasis in biological free radical research can generally be summarized as follows. H_2O_2 is produced in tissues as a result of metabolism, oxidant xenobiotics, or metal-catalyzed autoxidations (6,7). The H_2O_2 then reacts with loosely chelated transition metal, e.g., iron or copper, to generate the extremely oxidizing hydroxyl radical or some species with similarly extreme reactivity, which, in turn, produces chemical damage to biological structures by abstracting hydrogen atoms from molecules, leading to inactivation of thiol-dependent enzymes, fragmentation and oxidation of proteins and DNA, and initiation of lipid peroxidation (8,9). The initiation of lipid peroxidation has often been considered the proximal cause of cell damage (10). However, the nonenzymatic peroxidation of polyunsaturated fatty acids (lipid peroxidation) is not necessary for cell damage to occur and, indeed, may often be a consequence rather than a cause of cell damage (11,12). In circumstances in which lipid peroxidation is ascribed a causative role, it is essential to show that there is an increase in products of lipid peroxidation. Such products are commonly measured by thiobarbituric acid (TBA).

Srivastava and Ansari (13) provided evidence that butylated hydroxytoluene (BHT), which inhibits nonenzymatic lipid peroxidation, protects against galactose-induced cataracts by protecting lens membranes from oxidative damage. This evidence is consistent with the view that the lens contains substantial amounts of peroxidizable unsaturated lipid (14) and appears to support the hypothesis that oxidative stress contributes to experimental cataracts but some caution needs to be exercised. We have studied the effects of BHT and butylated hydroxyanisole (BHA) on galactose

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cataracts and of BHA on diabetic cataracts. Our studies showed no protective effect of BHT or BHA. BHT neither inhibited glutathione (GSH) nor protected against other biochemical alterations typically associated with sugar cataracts. Inhibition of galactose feeding and the induction of lens GSH by feeding high concentrations of antioxidants may be more likely explanations for the observed effects.

RESEARCH DESIGN AND METHODS

Wistar rats (150 g) were randomized into groups of six and observed for 3 days before the onset of feeding routines. The rats were then fed ad libitum a standard powdered laboratory chow containing 0.1% wt/wt BHA or 0.4% wt/wt BHT (Sigma, Poole, UK), with a minimum of six animals per group and nonspill food bowls. BHT was added to the diet 5 days before the addition of galactose in two groups of six animals each and at the same time as galactose in another group of six. Food intake (judged by measuring changes in food-bowl weight) and animal weight gain were measured. Galactose (Sigma) was added to the diet at 20% wt/wt for the BHA study and 50% wt/wt for the BHT study. Bulk was made up in the controls by the addition of Solkafloc cellulose (Johnson House, Wokingham, UK).

Diabetes was induced by administration of 50 mg/kg i.p. streptozocin in a citrate buffer, pH 4.5. Antioxidant was administered after diabetes was clearly established (at 72 h) because antioxidants are reported to inhibit the induction of diabetes (15). The presence of diabetes was confirmed by polyuria, glycosuria, and a plasma glucose concentration exceeding 15 mM at termination.

After 38 days, rats were killed by cervical dislocation under terminal anesthesia induced by injection of 2.5 mg/kg i.p. diazepam and 0.315 mg/kg i.m. Hypnorm phentanyl (Janssen, Oxford, UK). Lenses were examined under maximal mydriasis (induced by 2% wt/vol homatropine) with a hand-held ophthalmoscope. Severity of cataracts was assessed by two observers via a four-stage scale. Stage 0 was normal, stage 1 was slight vacuolation, stage 2 was vacuolation with cortical involvement, and stage 3 was evidence of a mature cataract (16). In cases of disagreement, the lower value was taken.

GSH measurements were taken via Ellman's reagent. The two lenses from each animal were pooled and homogenized by hand in 1 ml 25 mM–150 mM Tris-HCl buffer, pH 7, and centrifuged at 12,000 × *g* for 10 min. Five hundred microliters of supernatant was added to 500 μl 20% trichloroacetic acid (TCA), vortexed, and recentrifuged at 12,000 × *g* for 10 min. Four hundred microliters of the resulting protein-free supernatant was added to 1.8 ml 250 mM potassium phosphate buffer, pH 7.5, containing 125 μM 5,5′-dithiobis-2-nitrobenzoic acid. The absorbance was measured at 412 nm. TCA-soluble thiol was calculated with the extinction coefficient of Ellman's reagent with glutathione of 13,600 $M^{-1}cm^{-1}$.

Galactitol was measured in rat lenses by periodate oxidation of the polyol to formaldehyde in an assay described before (17) but adapted by condensation of the aldehyde with pentanedione and ammonium acetate (in the Hanzsch assay) to form methyltoluidine (18). Seventy-five microliters of 10% TCA was added to 125 μl centrifuged lens homogenate and then centrifuged at 12,000 rpm for 5 min. To 15 μl protein-free supernatant we added 50 μl 1 N HCl and 250 μl 25 nM $NaIO_4$. The mixture was incubated for 30 min at 37°C. At the end of this time, 50 μl 1.4 N NaOH and 50 μl 10% $ZnSO_4$ were added. The mixture was allowed to stand for a few minutes after vortexing before the addition of 500 μl 2 M ammonium acetate containing 20 mM pentanedione. Methyltoluidine (absorbance maximum 415 nm) was measured in the supernatant after incubation for 1 h at 37°C. Standards of polyol were treated in the same manner throughout, and recovery of galactitol in spiked lens homogenate samples was complete. Under these conditions, the method measures primarily galactitol, because this polyol is the major sugar component of the lens of the galactose-fed rat (19) and galactitol is oxidized to yield 2 M formaldehyde, compared with 1 M for galactose (18).

Lipid peroxidation in the lens was estimated by the use of TBA, which condenses with aldehydes resulting from lipid peroxidation (e.g., malondialdehyde [MDA]) to form a pink chromophore (20). To the insoluble membrane-protein fraction resulting from the homogenization and centrifugation of two lenses were added 200 μl 8.1% sodium dodecyl sulfate, 1.5 ml 20% acetic acid (adjusted to pH 3.5 with NaOH), and 1.5 ml 0.8% TBA (Sigma). Water was added to a final volume of 4 ml, and the resulting suspension was vortexed and incubated at 95°C for 1 h. At the end of this period, the mixture was cooled and centrifuged (3000 rpm for 10 min), and the absorbance of the supernatant was measured at 532 nm. Concentration of TBA-reactive aldehyde was estimated with the absorbance coefficient given for the MDA-TBA adduct, $1.4 \times 10^5\ M^{-1}cm^{-1}$ (10).

The TBA method assesses stable aldehydic products of lipid peroxidation, but there are some doubts about the usefulness and general validity of this widely employed assay for lipid peroxidation. TBA is nonselective with respect to reactive substances, which are not solely aldehydes derived from nonenzymatic lipid peroxidation. In addition, the TBA assay cannot be used to measure peroxides that do not decompose to aldehydes (e.g., MDA) of the general structure shown in Fig. 1. Therefore, it can not be used to measure H_2O_2 or other peroxides that might conceivably accumulate in biological systems. Frew et al. (21), in an assessment of different methods for the determination of low levels of peroxides (1–10 μM), referred to the potential sensitivity of the oxidation of Fe^{2+} by peroxides at low pH in the presence of the Fe^{3+}-complexing dye xylenol orange (Sigma). This method has been used to measure H_2O_2 produced in low-density lipoprotein after exposure to autoxidizing glucose (Hunt et al., this issue, p. 1420). Peroxide intermediates of lipid peroxidation, and peroxides potentially formed from other processes were estimated (in the protein-free supernatant) by the peroxide-mediated oxidation of Fe^{2+} to Fe^{3+} in 25 mM H_2SO_4 in the presence of xylenol orange, which forms a purple complex with the latter cation (absorbance maximum 560 nm, E = 14,000 $M^{-1}cm^{-1}$; 22). In one experiment,

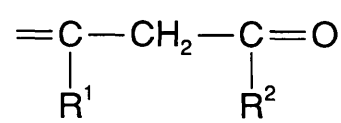

FIG. 1. General aldehyde structure.

10,000 U/ml catalase (Sigma) was incubated with lens homogenate before the addition of 5% TCA.

Loss of protein tryptophan fluorescence is associated with cataract formation and may be caused by oxidative damage (1). Ten microliters of the soluble protein fraction resulting from the homogenization and centrifugation of two lenses was added to 3 ml 3 M guanidine hydrochloride solution. Fluorescence was measured at an excitation wavelength of 280 nm and an emission wavelength of 350 nm after incubation of protein with guanidine hydrochloride for 30 min.

Statistical analyses were performed via pooled or separate variance *t* tests, analysis of variance, and χ^2-tests as appropriate (23). Data are presented as means ± SD.

RESULTS

Tolerance of phenolic antioxidants. Many feeding and toxicity studies of phenolic antioxidants have been performed on rats in the context of safety in the food industry (24). Such studies have shown that 0.4% BHT, the concentration used in the feeding study by Srivastava and Ansari (13), is at the limits of the acute toxicological tolerance of animals (25). Any suppression of feeding caused by the drug would have an obvious effect on the amount of galactose consumed. The precise nature of the dose-response relationship between galactose and cataracts is not completely understood, but it appears that there is a critical period during which a high dose of galactose must be consumed for the lens to be set on an irreversible path toward opacification (26).

We found that the addition of 0.4% BHT to the diet caused a marked cessation of feeding and was associated with weight loss in the rats (Fig. 2). Daily weight gains and food intake appeared to recover within 4 days after onset of BHT feeding, although weights never achieved those of the rats in the control group (Fig. 2). In the galactose feeding studies, 0.4% BHT was added to the diet 5 days before the addition of galactose to overcome the problem of suppressed galactose feeding. At day 38, body weights were 260 ± 10 g in control, 241 ± 18 g in BHT, 214 ± 18 g in galactose, and 199 ± 19 g in BHT and galactose rats.

Cataract formation. Rats were examined at days 10, 27, and 38 for the presence of cataracts. Our animals seemed somewhat more resistant to galactosemic cataracts than those used by Srivastava and Ansari (13) (probably because of strain or environmental differences) and showed only slight vacuolation of the lenses (stage 1) at day 10. There were no differences between the extent of this in the galactose-fed groups and those prefed BHT at this time. At day 27, cataracts had progressed to stage 2, but again, no significant differences were observed (Fig. 3). At day 38 (when the experiment was terminated and the animals were processed), cataracts had progressed in some animals to stage 3, but again, there were no differences between the animals fed galactose and prefed BHT before the addition of galactose to the diet. We did, however, observe at this stage that the animals administered BHT at the same time as galactose showed a small but significant inhibition of cataract formation ($P < 0.05$; Fig. 3). When the data from these animals were pooled with those from the BHT-prefeeding experiment, the significance of the inhibition by BHT at day 38 lost statistical significance because *P* rose to 0.12.

In our preliminary studies of BHA (which is as effective an inhibitor of lipid peroxidation as BHT), we found that this antioxidant was tolerated only at a level of 0.1% wt/wt in the diet, with marked suppression of feeding, inhibition of growth, and frank toxicity at higher concentrations. At this level in the diet, BHA had no protective effect against galactosemic or diabetic cataracts (Fig. 4). For diabetic cataracts, of course, suppression of feeding is not a complicating factor because cataracts result from diabetes, although food restriction might have some hyperglycemia-lowering effect.

GHS and galactitol levels in lens. The galactose-fed rats were killed on day 38, when some were progressing to stage 3 cataracts, a stage equivalent to those in the study by Srivastava and Ansari (13), where extensive protection by BHT was observed. We observed no protection against GSH loss in the BHT-prefed animals (Fig. 5). Similarly, 0.1% BHA did not protect against GSH loss in galactosemic or diabetic animals (control, 5.08 ± 0.16 μm/g wet wt; galactose, 1.56 ± 0.22 μm/g wet wt; 0.1% BHA, 5.52 ± 0.35 μm/g wet wt, $P < 0.05$ vs. control; BHA and galactose, 1.55 ± 0.17 μm/g wet wt, NS vs. galactose). BHT did not have an effect on galactitol concentrations in the lens (control vs. BHT, 17.4 ± 3.5 vs. 17.5 ± 4.9 nmol/mg wet wt), and it did not

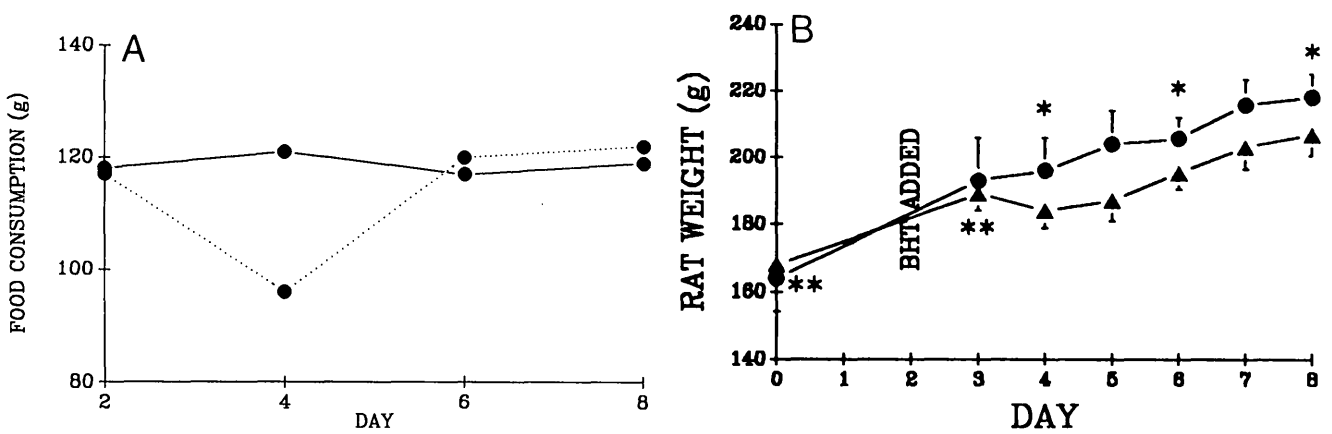

FIG. 2. Effect of 0.4% butylated hydroxytoluene (BHT) on food intake (per 6 rats; *A*) and rat individual weight gains (*B*). Day 0 is time when rats were switched from pellet to powdered chow. *A*: *solid line*, control diet; *dotted line*, diet with BHT added at day 2. *B*: changes in body weight when BHT was added to diet at day 2. ●, Control; ▲, 0.4% BHT. *$P < 0.05$. **NS.

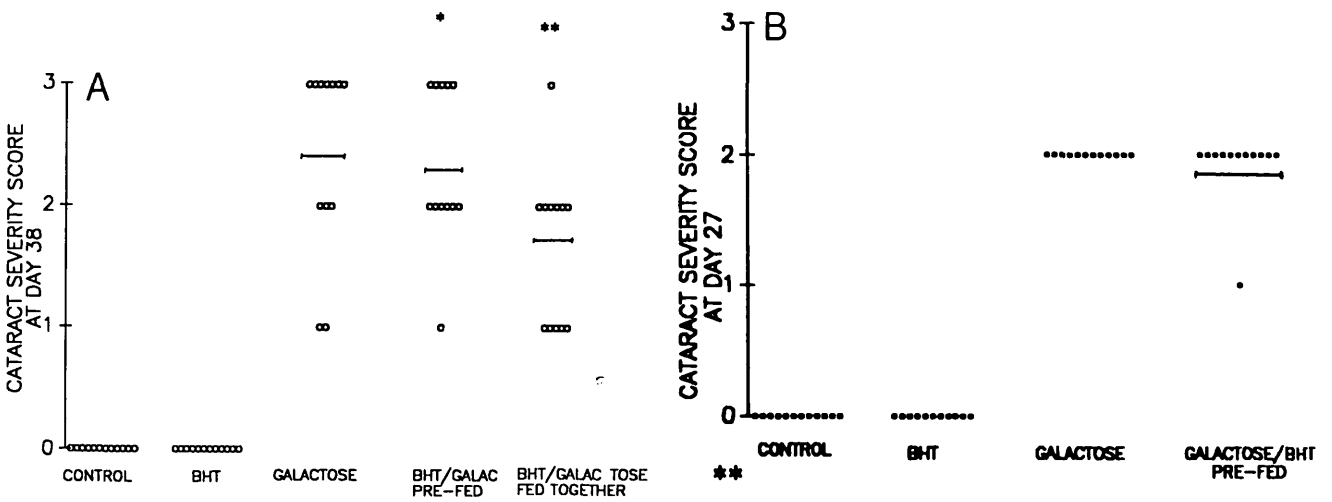

FIG. 3. Effect of butylated hydroxytoluene (BHT) on cataract development. Cataract was assessed as described in RESEARCH DESIGN AND METHODS. *A*: individual data points for each cataract scored. BHT was added to powdered chow 5 days before addition of galactose or at same time. *NS vs. galactose, $\chi^2 = 1.4$. **$P < 0.05$ vs. galactose, $\chi^2 = 1.3$. *B*: cataract severity scores at day 27.

protect against the loss of protein tryptophan fluorescence associated with cataract formation (control, 93.81 ± 10.00; galactose, 67.1 ± 12.0, $P < 0.05$ vs. control; BHT, 88.65 ± 10.00 NS vs. control; galactose and BHT, 60 ± 16, $P < 0.05$ vs. control). The decrease can be partially accounted for by loss of protein in the galactose-fed rats (control [$n = 6$] vs. galactose [$n = 6$], 17.8 ± 2.0 vs. 14.0 ± 2.0 mg/ml), but most of the fluorescence decrease appears linked to alterations in protein structure.

Indices of oxidative stress. If lipid peroxidation is a cause of galactosemic cataracts, then galactose should increase the amount of TBA-reactive material present in the lens, and BHT should inhibit such an increase. In contrast, we found that galactose suppressed the formation of TBA-reactive material (Fig. 6). This decrease was not exacerbated by BHT. This trend was not unique to the lens but was also observed in the liver, kidney, and erythrocytes (data not shown). There was an increase, however, in the galactose-fed animals, of a stable oxidizing agent detectable in the TCA supernatant by the oxidation of Fe^{2+} to Fe^{3+} in the presence of xylenol orange and H_2SO_4 (Fig. 7). However, this oxidant is not H_2O_2 present in the lens homogenate (at least not before addition of TCA) because catalase, which destroys this oxidant, did not diminish the signal. The level of this oxidant was not altered by BHT feeding (Fig. 7).

DISCUSSION

Oxidative stress can be defined as the production of free radicals at rates or sites exceeding those consistent with the maintenance of normal cellular function. Implicit in most current definitions of oxidative stress is the idea that free radicals cause chemical damage to sensitive cellular components. Unfortunately, unequivocal measurements of such oxidative chemical damage do not exist (27). Even a measurement of lipid peroxidation via the straightforward TBA method is only an indication that lipid peroxidation has occurred because of some process, which could include cell death. Prevention of cell damage by selective inhibitors of lipid peroxidation

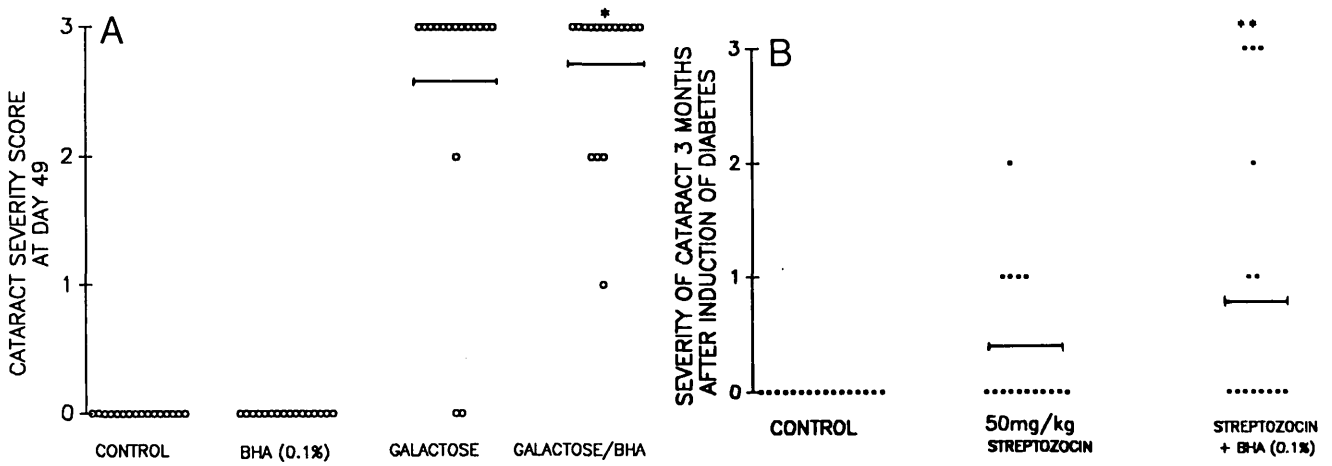

FIG. 4. Effect of butylated hydroxyanisole (BHA) on galactosemic and diabetic cataracts. Cataracts were assessed on scale of 0–4, where 0 was normal, and extra stage was employed between stages 2 and 3 of previous observation strategy. *A*: individual data points for each cataract scored. Galactosemic cataracts were induced by feeding 20% galactose and were scored after 7 wk. *NS vs. galactose. *B*: data for streptozocin-induced diabetic cataract, where antioxidant was administered after diabetes was established, and cataracts were scored after 3 mo. **NS vs. streptozocin.

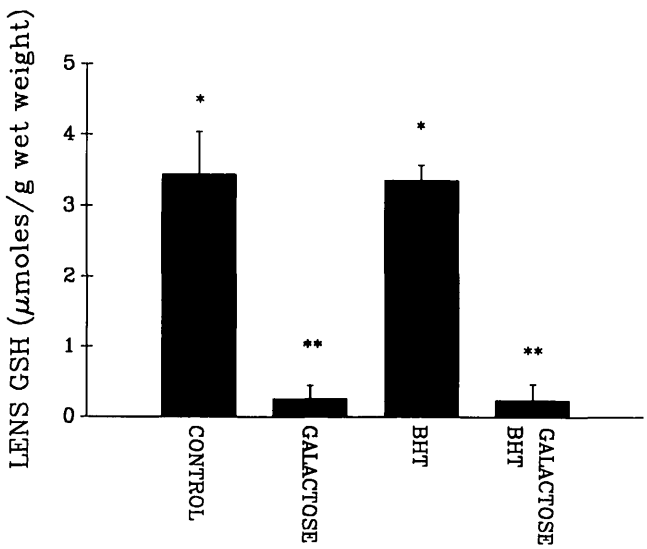

FIG. 5. Glutathione (GSH) alterations in lenses of rats fed galactose and butylated hydroxytoluene (BHT). GSH was measured as described in RESEARCH DESIGN AND METHODS. *n* = 6/group. *NS vs. galactose and galactose and BHT. **NS vs. control and BHT.

must be combined with measurement of enhanced TBA reactivity before a causative role for the process can be unequivocally endorsed.

We found that lipid-soluble inhibitors of lipid peroxidation do not inhibit sugar-cataract formation. When BHT and galactose together caused a slight suppression of cataractogenesis, we believed the suppression was probably explained by inhibition of feeding caused by the drug and thus loss of accumulation of the galactose, perhaps at a critical stage. Although the effect on food intake was transient, it is conceivable that a continuous stress of galactose is required for a cataract to progress. Even a transient decrease in galactose intake at a critical stage of cataractogenesis could have a significant delaying effect. It is possible that a similar effect occurred in the experiment reported before, but unfortunately, neither food intake nor body weight changes were reported (6). The inhibition of cataractogenesis by BHT reported by Srivastava and Ansari (13) might also have resulted from the small induction of lens GSH apparently caused by BHT in that strain of rat. BHT is metabolized by cytochrome P_{450} and glutathione transferase, and induction of these activities and GSH has been reported in some animals (28). No induction of lens GSH (Fig. 4) or liver P_{450} (data not shown) was observed in our rats. However, GSH depletion appears to be an important component of cataractogenic lens changes. Thus, drugs that induce this lens constituent, as a consequence of their own metabolism, could be anticipated to inhibit cataract formation. This could not be taken as a direct antioxidative protective effect of the drug against cataracts. It is also unclear whether the phenolic antioxidants actually penetrate the lens. It is reasonable to assume that they can, however, because BHT penetrates other tissues (24), and the lipid-soluble antioxidant vitamin E is also present in the lens. Furthermore, the cataractogenic lesion initiated by galactose may not only be caused by a direct effect of galactose on the lens of the whole animal; it may also possess a systemic component that causes secondary damage to the lens. These are problems that still need to be addressed in assessing the oxidative-stress hypothesis of galactosemic and other cataract formation.

If galactose feeding is associated with enhanced nonenzymatic lipid peroxidation in the lens, then this peroxidation should be measurable. Levels of lipid peroxidation were not reported by Srivastava and Ansari (13), but we found that galactose actually causes a decrease in the formation of stable TBA-measurable aldehydic products of lipid peroxidation rather than an enhancement. This is consistent with the apparent lack of effect of BHT on galactosemic cataract formation and also with a report that vitamin E (another phenolic inhibitor of lipid peroxidation) had no effect on progression of cataracts in this model (29). However, this is by no means conclusive evidence that oxidative stress plays no role in galactosemic cataracts. Indeed, we found

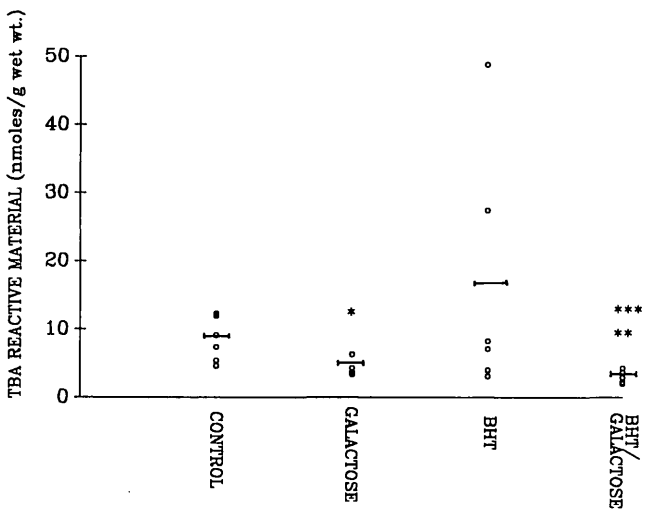

FIG. 6. Thiobarbituric acid (TBA)-reactive material in rat lenses, measured as described in RESEARCH DESIGN AND METHODS. Results are expressed as individual data points for each animal studied. High *error bar* in butylated hydroxytoluene (BHT) sample is result of 1 animal possessing surprisingly high levels of TBA-reactive material. *$P < 0.05$ vs. control. **NS vs. galactose. ***$P < 0.05$ vs. control.

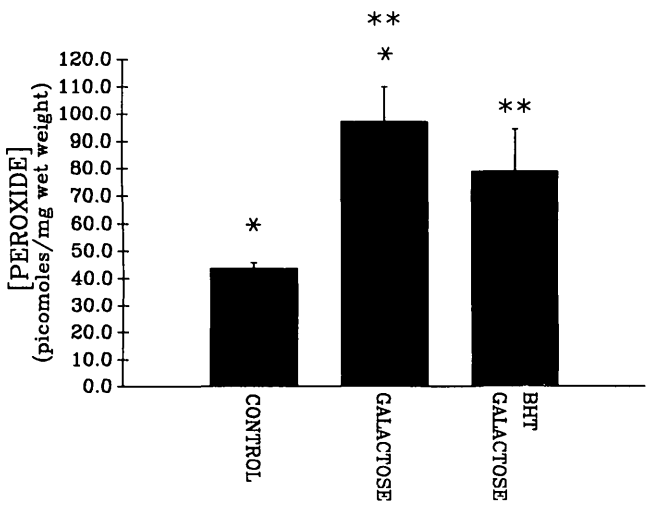

FIG. 7. Fe^{2+} oxidant in protein-free lens supernatant. Material able to oxidize Fe^{2+} to Fe^{3+} was assessed with xylenol orange as described in RESEARCH DESIGN AND METHODS. Identity of this species is not known but termed *peroxide*. *$P < 0.05$ vs. butylated hydroxytoluene and galactose. **NS vs. control.

that the lens of a galactose-fed rat contains increased levels of a TCA-soluble substance that is capable of oxidizing Fe^{2+} to Fe^{3+} in dilute acid. This unknown oxidant is probably a peroxide because it appears to be relatively stable, but it certainly does not have H_2O_2 present before addition of TCA, and its formation is not suppressed by feeding a phenolic inhibitor of lipid peroxidation. Its identity remains unknown.

It is important to recognize that the absence of BHT-inhibitable TBA-measurable nonenzymatic lipid peroxidation does not imply absence of oxidative stress. It is often poorly recognized that TBA can be nonselective with respect to reactive substances, which may be derived from prostaglandin metabolism or sugar degradations or from nonenzymatic lipid peroxidation (30). Indeed, TBA-reactive material in a normal rat lens has been suggested to be a poor measure of nonenzymatic lipid peroxidation (31). Such ambiguity may provide an explanation for the higher levels of TBA-reactive material present in two of the BHT-fed animals studied, if, for example, they are related to prostaglandin metabolism, which might be quite variable within animals. Perhaps most important, the TBA assay cannot be used to measure peroxides that do not decompose to aldehydes similar in structure to MDA, the major aldehydic product of nonenzymatic lipid peroxidation (32). Peroxides from other sources might thus be overlooked.

We are led to conclude that enhanced nonenzymatic lipid peroxidation leading to the production of TBA-reactive material is not a causative factor in galactosemic or diabetic cataractogenesis, although some alteration of lens redox status and oxidant accumulation, which might be deleterious, is present. In those cases where an inhibitor of nonenzymatic lipid peroxidation inhibits pathological changes, it must be demonstrated that the pathology is associated with increased lipid peroxidation and that the inhibitor does not influence dietary habits or induce detoxification systems.

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