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On the mechanisms of induction of cancer-protective enzymes: A unifying proposal

(antioxidants/azo dyes/murine hepatoma cells/hydroquinones/quinone reductase)

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ABSTRACT **Induction of detoxification enzymes is a major mechanism whereby a wide variety of chemical agents protect rodents against neoplastic, mutagenic, and other toxicities of carcinogens. The enzyme NAD(P)H:(quinone acceptor) oxidoreductase (EC 1.6.99.2) can protect against the toxicities of quinones and is a useful marker for protective enzyme induction. Quinone reductase can be induced in murine Hepa 1c1c7 hepatoma cells and 3T3 embryo fibroblasts by compounds that are chemoprotectors *in vivo*, including some phenolic antioxidants, azo dyes, aromatic diamines, and aminophenols. Structurally dissimilar catechols (1,2-diphenols) and hydroquinones (1,4-diphenols) induce quinone reductase in these systems, but resorcinol (1,3-diphenol) and its substituted analogues are inactive. Futhermore, only aromatic 1,2- and 1,4-diamines and aminophenols are inducers, whereas the 1,3-diamines are completely inactive. These findings suggest that the functional capacity to form quinones or quinonediimines, rather than the precise structure, is essential for inductive activity and that the generation of the signal for enzyme induction depends upon oxidation–reduction lability. The observations that some chemoprotective compounds (e.g., azo dyes, β-naphthoflavone) induce both cytochromes P-450 and quinone reductase, whereas others (e.g., *tert*-butylhydroquinone) induce only quinone reductase, can be reconciled by the fact that inducers of the first type are metabolized by P-450 enzymes to form products that are functionally similar to compounds of the second type.**

A surprising variety of chemicals protect rodents against neoplastic, mutagenic, and other toxic effects of many types of carcinogens (1, 2). A striking feature of the chemoprotectors is that they belong to totally unrelated chemical classes such as substituted phenols, azo dyes, coumarins, sulfur compounds (disulfiram, isothiocyanates, carbon disulfide), flavones, indoles, retinoids, tocopherols, and selenium compounds (3). Some of these protective substances alter the metabolic fate of carcinogens (4–6) by modulating the activities of either or both phase I and phase II drug-metabolizing enzymes[†] (7–15). Induction of enzymes that detoxify electrophilic metabolites of carcinogens may interrupt the neoplastic process. Thus, BHA [3(2)-*tert*-butyl-4-hydroxyanisole], a phenolic antioxidant, which is a widely used food additive and protects against many carcinogens, enhances the hepatic and peripheral activities of several detoxification enzymes, including glutathione *S*-transferase, NAD(P)H:quinone reductase, epoxide hydrolase, and glucuronide-conjugating systems, and raises the levels of glutathione and enzymes concerned with its reduction (2, 7–10, 14–16).

How do so many structurally unrelated compounds induce similar chemoprotective enzymes? We approached this question by examining the relation between structure and inductive activity among three classes of enzyme inducers and chemoprotectors: phenolic antioxidants, aromatic diamines, and azo dyes. Closely related structures within each class are either susceptible or resistant to facile oxidation–reduction reactions (Fig. 1). Thus, 1,2-diphenols (catechols) or 1,2-diamines and 1,4-diphenols (hydroquinones) or 1,4-diamines participate in reversible conversions to quinones or quinonediimines, respectively, whereas this is not possible for 1,3-diphenols (resorcinols) or 1,3-diamines. These agents were tested as enzyme inducers in a murine hepatoma cell line (Hepa 1c1c7) that mimics the response to such compounds of a variety of mouse tissues *in vivo* (17–19). 3T3 mouse embryo fibroblasts were also used in some studies. Cytosolic NAD(P)H:quinone oxidoreductase (EC 1.6.99.2; also known as menadione reductase, DT diaphorase, or vitamin K reductase) activities were used as a measure of induction, since this enzyme plays a key role in protecting cells against the toxicities of a variety of quinones (9, 20, 21) and is induced coordinately with other protective enzymes in rodents (22–24). Our results suggest that the capacity for facile oxidation–reductions is a common and obligatory property of these types of phase II enzyme inducers.

MATERIALS AND METHODS

Treatment of cells. Hepa 1c1c7 cells (a gift of J. P. Whitlock, Jr., Stanford University) were grown in alpha minimal essential medium minus nucleosides (GIBCO) plus 10% fetal calf serum. Swiss albino mouse embryo fibroblasts (3T3 cells; American Type Culture Collection, CCL 92) were grown in Dulbecco's modified Eagle's medium (GIBCO) with high glucose and 10% calf serum. Cells were grown in 5% CO_2 at 37°C.

For our standard protocol, Hepa 1c1c7 or 3T3 cells were seeded at 0.89×10^6 cells per 10-cm plate, grown for 48 hr to late log phase, and exposed, usually for 24 hr, to fresh medium containing test compounds in dimethylsulfoxide (0.1% by vol). Hepa or 3T3 cells were washed three times with 10 ml of cold 0.25 M sucrose or 0.25 M sucrose/10 mM potassium phosphate, pH 7.2, respectively, collected from the plates by scraping, frozen in liquid nitrogen, and stored at −80°C until enzyme activities were assayed.

Preparation of Cytosol Fractions. Thawed sucrose suspensions of Hepa 1c1c7 cells were homogenized at 0–4°C and then were centrifuged at 5000 × *g* for 20 min. The supernatant

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Abbreviation: BHA, 3(2)-*tert*-butyl-4-hydroxyanisole.

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[†]The enzymes involved in the metabolism of xenobiotics have been classified into two broad categories. Phase I enzymes (which include the cytochromes P-450) functionalize compounds by oxidation, reduction, or hydrolysis, whereas phase II enzymes carry out the conjugations of functionalized compounds with endogenous ligands (e.g., glucuronic acid, glutathione, and sulfate). We classify quinone reductase as a phase II enzyme, since it does not introduce new functional groups and is often induced coordinately with conjugation enzymes.

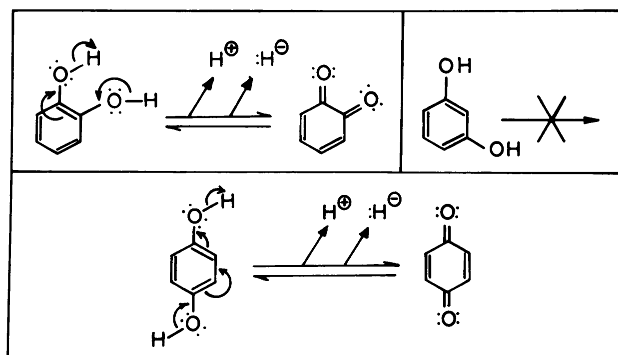

FIG. 1. Oxidation–reduction reactions of diphenols. Catechols (1,2-diphenols, *Upper Left*) and hydroquinones (1,4-diphenols, *Lower*) can undergo facile reversible oxidations to the corresponding quinones, whereas resorcinol (1,3-diphenol, *Upper Right*) cannot give rise to quinones; a balanced 1,3-quinone structure cannot be written.

fluids received 0.2 volume of 0.1 M $CaCl_2$/0.25 M sucrose and, after 30 min at 4°C, were centrifuged at 15,000 × *g* for 20 min to provide the cytosols. 3T3 cells were sonicated for 10 sec and then were centrifuged at 17,000 × *g* for 20 min to give cytosols.

Determination of Quinone Reductase. The rate of reduction of 2,6-dichloroindophenol (40 μM) by NADH (200 μM) in a 3-ml assay system at pH 7.4 was measured at 600 nm (9). Specific activities are expressed as nmol of 2,6-dichloroindophenol reduced per min per mg of protein (25).

Synthesis and Characterization of Compounds. Compounds **2–5** (Fig. 2) have been described (26). 4-*tert*-Butylresorcinol was synthesized from *tert*-butyl alcohol and resorcinol (27). Resorcinol and 4,6-di-*tert*-butylresorcinol (Aldrich) were recrystallized from toluene, and 3-5-di-*tert*-butylcatechol (Aldrich) from *iso*-octane. 1,3-Phenylenediamine hydrochloride (Aldrich) was converted to the sulfate and recrystallized from ethanol. Azo dyes were a gift from C. Huggins and J. Pataki (The University of Chicago). All other compounds (Aldrich) were used as received.

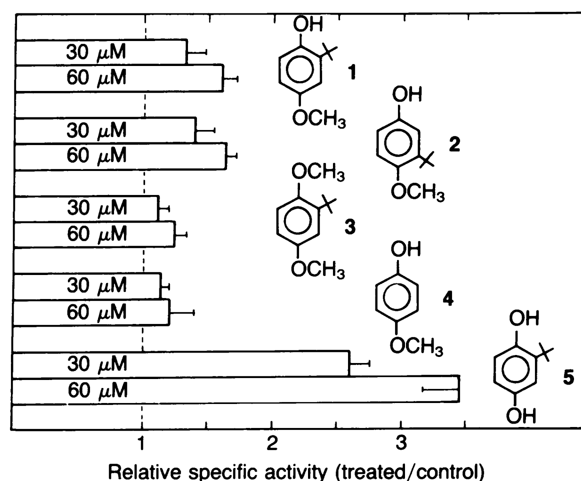

FIG. 2. Induction of quinone reductase in Hepa 1c1c7 cells by the substituted phenols 3-*tert*-butyl-4-hydroxyanisole (**1**, major isomer of BHA), 2-*tert*-butyl-4-hydroxyanisole (**2**, minor isomer of BHA), methyl ether of *tert*-butyl-4-hydroxyanisole (**3**), 4-hydroxyanisole (**4**), and *tert*-butylhydroquinone (**5**). The compounds (30 and 60 μM) were added after 48 hr of growth, and cells were harvested 24 hr later. The cytosolic quinone reductase activities (means of 4 determinations ± SEM) are expressed as the ratios of treated to untreated controls. Mean control value: 485 ± 31 nmol per min per mg of protein. The error bars shown for each set of ratios have been divided by the mean control values.

RESULTS

Phenolic Antioxidants. Many hindered phenols are inducers of detoxifying enzymes *in vivo* and protect against chemical carcinogens (14, 15, 22, 23, 28). Fig. 2 shows that in Hepa 1c1c7 cells 30 and 60 μM concentrations of the major and minor isomers of BHA (structures **1** and **2**), the methyl ether of BHA (**3**), 4-hydroxyanisole (**4**), and *tert*-butylhydroquinone (**5**) all induced quinone reductase, although compounds **3** and **4** were only slightly (but significantly) active. *tert*-Butylhydroquinone (**5**) was the most active inducer. Since Hepa 1c1c7 cells contain *O*-dealkylase activity (29), the modest inductive activity of compounds **1–4** may result from dealkylation products.

Major structural modifications of *tert*-butylhydroquinone by introduction of various types of *O*-alkyl groups did not greatly affect inductive activity *in vivo* (22–24). Since *tert*-butylhydroquinone is a major metabolite of BHA, it may be the common active form of these compounds (30). The relative potencies of induction of alkylated congeners of *tert*-butylhydroquinone may therefore reflect rates of dealkylation or transport.

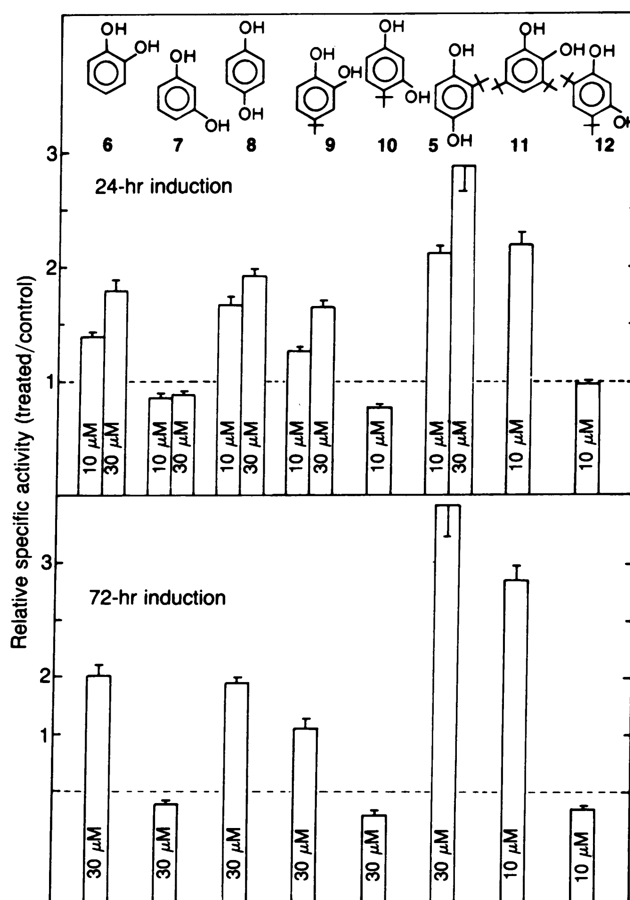

FIG. 3. Induction of quinone reductase in Hepa 1c1c7 cells by catechol, resorcinol, and hydroquinone derivatives: **6**, catechol; **7**, resorcinol; **8**, hydroquinone; **9**, 4-*tert*-butylcatechol; **10**, 4-*tert*-butylresorcinol; **11**, 3,5-di-*tert*-butylcatechol; **12**, 4,6-di-*tert*-butylresorcinol. The experiment was conducted as described in the legend to Fig. 2, except that the cells were exposed to 10 or 30 μM concentrations of the compounds for 24 hr (*Upper*) or 72 hr (*Lower*). Mean control values: 337 ± 11 and 302 ± 10 nmol per min per mg, for the 24-hr and 72-hr inductions, respectively.

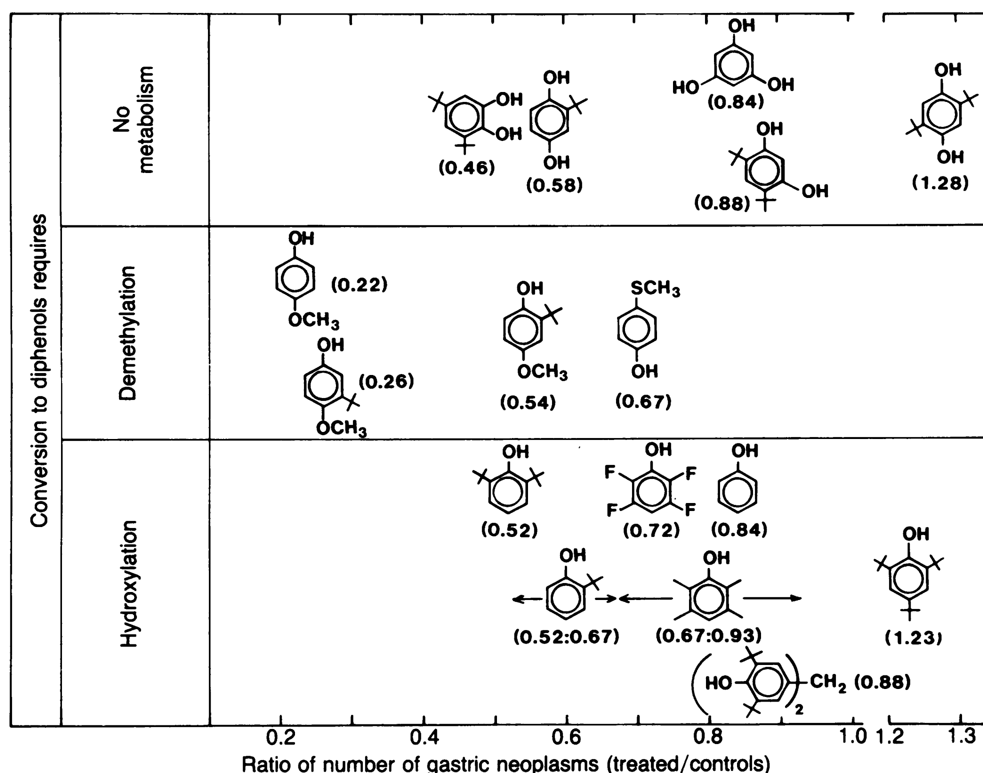

FIG. 4. Protection by various phenols against benzo[a]pyrene-induced neoplasia of the forestomach in female ICR/Ha mice. The results of Wattenberg *et al.* (28) are arranged as potency ratios (in parentheses) of the number of gastric neoplasms in treated mice to the number in untreated mice. In those cases where two experiments were done, both ratios are shown and the range is indicated by arrows. The compounds are also arranged into three groups to indicate the metabolic conversion required to form diphenols: i.e., no metabolism, demethylation, or hydroxylation.

Examination of the inductive capacity of eight diphenols in Hepa 1c1c7 cells (Fig. 3) has shown that (*a*) the *tert*-butyl group is not essential since hydroquinone (compound **8**) and catechol (**6**) were inducers (compare **6** with **9** and **11** and compare **8** with **5**) and that (*b*) strikingly, no 1,3-diphenol (**7**, **10**, or **12**) was an inducer, even when added to the cultures on three successive days (Fig. 3). Thus, in this family of compounds the presence, absence, or positions of *tert*-butyl groups are far less important than the relative positions of the phenolic hydroxyl groups; 1,3-diphenols are invariably inactive as inducers. Comparable results were obtained with 3T3 fibroblasts, in which 30 μM concentrations of only 1,2- and 1,4-diphenols but not 1,3-diphenols acted as inducers.

Do these enzyme-induction patterns correlate with the ability of phenolic antioxidants to protect against chemical carcinogenesis? Wattenberg *et al.* (28) have described a series of phenols that protect against benzo[a]pyrene-induced neoplasia in the forestomach of female ICR/Ha mice. Fig. 4 shows the protective potency of 16 pertinent phenols, which are arranged into three groups: diphenols and compounds which can be converted to diphenols by either demethylation or hydroxylation. Except for 2,5-di-*tert*-butylhydroquinone(which is not protective), all significant protectors are catechols or hydroquinones or may be easily converted to such structures by metabolic reactions, whereas the 1,3-diphenols (resorcinols) are weakly if at all protective. Therefore, the ability to induce quinone reductase in two cell lines correlates almost perfectly with protection against benzo[a]pyrene-induced gastric neoplasia. The presence, absence, or position of the *tert*-butyl groups is also unimportant for protection, whereas the relative positions of the phenolic hydroxyl groups are critical.

Aromatic Diamines and Aminophenols. These compounds are of interest for two reasons. First, in analogy to the corresponding diphenols, the 1,2- and 1,4-diamines undergo facile oxidations to quinonediimines, whereas the 1,3-diamines resist such oxidation. Second, aromatic diamines and aminophenols are metabolic products of azo dyes, which are potent inducers of quinone reductase (see below). At 30 μM concentrations, 1,2-phenylenediamine (Fig. 5, com-

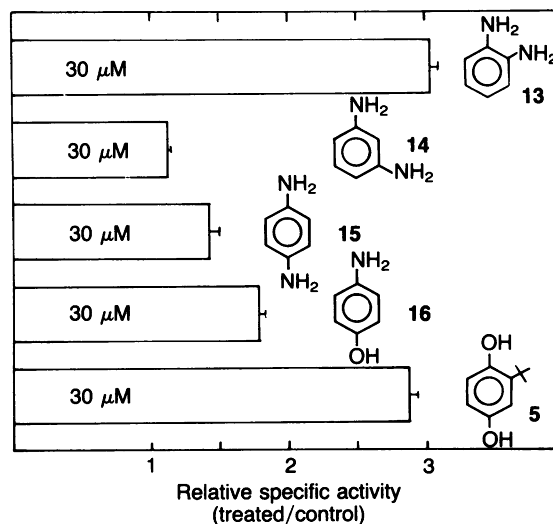

FIG. 5. Induction of quinone reductase in Hepa 1c1c7 cell cytosols by 1,2-phenylenediamine (**13**), 1,3-phenylenediamine (**14**), 1,4-phenylenediamine (**15**), 4-aminophenol (**16**), and *tert*-butylhydroquinone (**5**). The experiment was conducted as described for Fig. 2. The cells were exposed to 30 μM concentrations of the compounds for 24 hr. Mean control value: 307 ± 4 nmol per min per mg.

pound **13**) and *tert*-butylhydroquinone (**5**) were equally effective inducers. 1,4-Phenylenediamine (**15**) and 4-aminophenol (**16**) were also active, but, in marked contrast, 1,3-phenylenediamine was completely inactive. As with diphenols, the ability for aromatic diamines to undergo oxidation–reduction reactions is essential for induction. Furthermore, in Hepa 1c1c7 cells, 1-amino-2-naphthol (10 μM) and 1,2-naphthoquinone (20 μM) elevated quinone reductase specific activities to twice control values.

Azo Dyes. Sudan III [1-(4-phenylazophenylazo)-2-naphthol] and related azo dyes potently protect rats against 7,12-dimethylbenz[a]anthracene-induced mammary cancer, leukemia, adrenal hemorrhage, and chromosomal aberrations in bone marrow (26, 31, 32). A number of azo dyes resemble polycyclic aromatic hydrocarbons in inducing both phase I enzymes (cytochrome P-448-dependent monooxygenases) and phase II enzymes (quinone reductase, UDP-glucuronyltransferase, glutathione *S*-transferase, and aldehyde dehydrogenase) (11, 12).

Exposure of Hepa 1c1c7 cells for 24 hr to 2 μM 1,1′-azonaphthalene (compound **17**), Sudan I (**18**), and Sudan III (**19**) resulted in 4- to 12-fold inductions of quinone reductase (ref. 19 and Fig. 6). Although the coordinate inductions of phase I and II enzymes by azo dyes have been attributed to the ability of these compounds to bind avidly to the aromatic-hydrocarbon receptor (Ah), it is unclear whether other mechanisms may also be involved. Aromatic azo dyes are extensively and rapidly metabolized by ring hydroxylation and by reductive cleavage of the azo linkages (33). Reductions of azo linkages in mouse and rat liver microsomes are largely CO-inhibited and their rates parallel the levels of cytochrome P-450 (34). In the Hepa 1c1c7 system, various azo dyes are metabolized rapidly to colorless products. Incubation of Hepa 1c1c7 cells with radioactive Sudan I gave rise to polar cleavage products with chromatographic properties similar to 4-aminophenol and 1-amino-2-naphthol. These products of Sudan I metabolism are both inducers of quinone reductase (see above) and are metabolites in animal tissues (33). Thus, azo dyes may act as inducers by undergoing metabolic cleavage to aminophenols, which are susceptible to oxidation–reductions similar to those in which 1,2- and 1,4-diphenols and diamines participate.

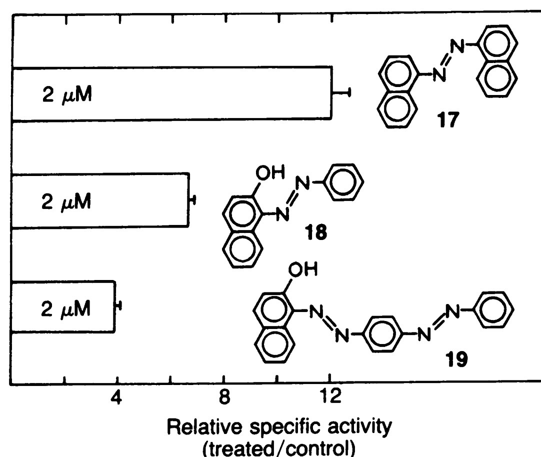

FIG. 6. Induction of cytosolic quinone reductase in Hepa 1c1c7 cells by azo dyes: **17**, 1,1′-azonaphthalene; **18**, 1-phenylazo-2-naphthol (Sudan I); **19**, 1-(4-phenylazophenylazo)-2-naphthol (Sudan III). The experiment was conducted as described for Fig. 2. The cells were exposed to 2 μM concentrations of the azo dyes for 24 hr. Control value: 274 ± 22 nmol per min per mg.

DISCUSSION

Nature of the Inductive Signal. Among the classes of compounds examined in detail (diphenols, diamines, and azo dyes), the capacity to generate species that can undergo oxidation–reduction reactions appears to be a key requirement for signaling the induction of quinone reductase. That is, in 1,2- and 1,4-disubstituted aromatics, the atomic nature of the substituents is probably of minor importance provided they can form single or double bonds with the aromatic ring. Although we have concentrated on a few classes of enzyme inducers, the proposed mechanism may reconcile the inducing activity of a wide range of compounds (e.g., daunorubicin, disulfiram, and related sulfur compounds; isothiocyanates; dithiolthiones; and polycyclic aromatic hydrocarbons) that not only induce quinone reductase in various systems (9, 35–37) but may also participate in a variety of oxidation–reduction reactions. Introduction into cells of extraneous oxidation–reduction-labile systems that have the capacity to undergo both 1- and 2-electron valency changes may lead to the generation of reactive oxygen species and/or perturb the balance of oxidation–reduction substrate (e.g., glutathione or nicotinamide nucleotides) pools at critical intracellular sites (21, 38).

Can signals arising from oxidation–reduction reactions be sufficiently selective to serve regulatory functions? Several examples of reprogramming of protein synthesis in response to apparently nonspecific stimuli are known: (*a*) induction of superoxide dismutase in *Escherichia coli* by oxidative stress (39); (*b*) induction of heat-shock proteins in many eukaryotic systems under adverse conditions, including oxidative stress (40); and (*c*) synthesis of reparative proteins in response to DNA damage by physical or chemical agents (41).

Mechanism of Induction and Possible Relation to Ah Receptor. Azo dyes, polycyclic aromatic hydrocarbons, β-naphthoflavone, and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin induce both phase I and phase II drug-metabolizing enzymes (12, 13, 42, 43). These planar aromatic compounds bind avidly to the protein product of the *Ah* locus and thereby enhance transcription of P-450 genes and metabolism of xenobiotics (43, 44). Although it has been suggested, primarily on genetic grounds, that the same receptor-mediated process for enhancing P-450 activity also participates in the induction of phase II enzymes (11–13, 43, 45, 46), an obligatory association of these two types of inductions is by no means established. Thus, phenolic antioxidants are highly effective inducers of phase II enzymes but produce only minor and variable effects on monooxygenase activities (16, 22). Participation of the Ah receptor in these phase II enzyme inductions by phenolic antioxidants is therefore unlikely. For this reason, we suggest that the coordinate induction of phase I and II enzymes by some compounds such as azo dyes may be associated with P-450 inducibility not only because both involve the same receptor-mediated process, but also because metabolism by cytochromes P-450 converts these compounds into species that may function as inducers for phase II metabolic enzymes (i.e., compounds that can undergo oxidation–reduction reactions of the type proposed) (Fig. 7). Several lines of evidence support this suggestion. Benzo[a]pyrene has been shown to be metabolized to several highly toxic and redox-labile quinones (47); increases in phase II enzyme activities usually lag behind the enhancement of monooxygenase activity (45, 46, 48); and administration of SKF 525 A (a monooxygenase inhibitor) renders benzo[a]pyrene less competent to induce quinone reductase (49). Furthermore, rates of cleavage of azo linkages are correlated with the monooxygenase content of microsomes (35), and this reaction gives rise to compounds that are oxidation–reduction-labile. The proposal that phase I enzymes convert complex organic compounds into species that

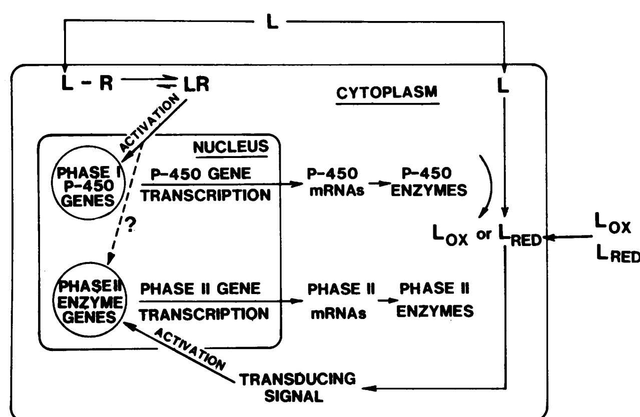

FIG. 7. Proposal for the interrelationship of induction of phase I and phase II drug-metabolizing enzymes. The inducing ligand (L) enters the cell and binds to the Ah receptor (R). The ligand–receptor complex activates the transcription of cytochrome P-450 genes. Cognate mRNAs are translated and active P-450 enzymes then metabolize the inducer L to either an oxidized form (L_{OX}; e.g., by hydroxylation or demethylation) or a reduced form (L_{RED}; e.g., by reduction of azo dyes to diamines or aminophenols). L_{OX} or L_{RED} is both reduction–oxidation-labile and produces an unknown signal that activates transcription of genes coding for phase II enzymes. Alternatively, if cells are exposed to appropriately redox-labile L_{OX} or L_{RED} directly, the Ah receptor may not participate in induction of phase II enzymes, and P-450 levels will not be elevated.

function as inducers for phase II enzymes is compatible with the existence of substances that are already functionally active inducers of phase II enzymes but are incompetent to bind to the *Ah* gene product.

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