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JOURNAL OF THE RETICULOENDOTHELIAL SOCIETY
Vol. 30, No. 5, November 1981

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Inhibition of Lymphocyte Transformation and Microtubule Assembly by Quinone Metabolites of Benzene: Evidence for a Common Mechanism

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Received March 24, 1981

Hydroquinone inhibits microtubule assembly and suppresses lectin-induced transformation of lymphocytes. Other workers have established a role for microtubules in modulating cell response to blastogenic stimuli. Several lines of evidence suggest that a) suppression of lymphocyte activation by hydroquinone is mediated through interference with the cytoskeleton, specifically, inhibition of microtubule function, b) inactivation is an SH-dependent process, and c) *p*-benzoquinone is the most likely molecular species responsible for these events.

INTRODUCTION

Benzene is an important industrial chemical which is toxic to bone marrow and lymphoid cells. Repeated exposure to benzene in experimental animals results in lymphocytopenia (23, 24, 41, 52, 58), a decrease in bone marrow cellularity (23), alterations in cell cycle kinetics of proliferating bone marrow precursor cells, and chromosomal aberrations (23, 27, 40, 53). Toxicity to bone marrow and lymphoid organs correlates with the concentration of the 2 principal dihydroxy metabolites of benzene accumulating in these tissues, hydroquinone (HQ) and catechol (CT) (17, 18). We have recently reported that HQ inhibits lectin-stimulated lymphocyte activation in culture (44) and interferes with microtubule assembly and tubulin-colchicine-binding activity (TCBA) *in vitro* (25). Both of these phenomena appear to be mediated through interaction with sulfhydryl (SH) groups: in the first case, with intracellular SH groups critical for lymphocyte agglutination and blastogenesis, and in the second, with SH groups necessary for maintaining microtubule functional integrity. An impressive body of evidence suggests that these 2 processes are directly related.

Microtubules, microfilaments, and their associated proteins are known to be involved in the regulation of cell surface receptor movement and cell growth (10, 36). Lectin-stimulated lymphocyte activation is a microtubule-dependent process; microtubule-disruptive agents enhance lymphocyte blastogenesis at low concentrations (43, 51, 61) but inhibit mitogenesis at higher concentrations (10, 16, 19, 46,

51, 56). SH group-specific reagents, such as N-ethylmaleimide (NEM), cytochalasin A, 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), and *p*-chloromercuribenzenesulfonate, inhibit microtubule self assembly and TCBA *in vitro* (20, 21, 28, 38). Of these reagents, those which penetrate the cell membrane (NEM, cytochalasin A) disrupt a variety of immune cell functions requiring cytoskeletal-dependent conformational change (11, 14, 55). Colchicine must also enter the cell to affect motility and surface receptor movement (2).

The mechanism of SH involvement in the self assembly of microtubules is unknown; however, studies have established the importance of free thiol groups in maintaining the functional integrity of microtubules (22, 28, 33, 38). Purified tubulin contains between 8 and 11 titratable SH groups, interaction with 2 or more of which results in inhibition of microtubule assembly (22, 28, 33, 38). Mann *et al.* (33) reported that 2 SH groups demonstrate statistically greater reactivity with NEM, a SH-alkylating reagent, than other free thiol groups on the protein and that the identical SH groups are associated with GTP binding to the tubulin molecule. Studies in this laboratory have demonstrated that HQ inhibits microtubule self assembly and enhances the decay of TCBA *in vitro* (25). Addition of GTP ameliorates the latter effect in a concentration dependent manner.

Hydroquinone and *p*-benzoquinone (*p*-BQ), the oxidation product of HQ, suppress lectin-stimulated lymphocyte blastogenesis *in vitro* at noncytotoxic concentrations (μ M) and, under physiologic conditions, HQ autoxidizes to form *p*-BQ, presumably forming the semiquinone radical in the reaction (26). In this scheme, the suppression of lymphocyte activation or cytotoxicity could be mediated through the formation of superoxide anion, *p*-semiquinone or direct alkylation by *p*-BQ. The SH reactivity of the quinones is well known (39) and has been demonstrated in the context of reactions involving 6-hydroxydopamine and related compounds (5, 15, 31, 48). In this paper, the effects of the polyhydroxy-metabolites of benzene on microtubule assembly are characterized and correlated with their effects on cytoskeletal-dependent lymphocyte activation in culture. A molecular mechanism is proposed for the modulation of lectin-induced lymphocyte blastogenesis by quinone compounds.

MATERIALS AND METHODS

Isolation of Rat Spleen Lymphocytes. Spleens pooled from at least 4 male Fischer-344 rats (Charles River) 6 to 8 weeks old were minced, homogenized gently with a loosely fitting Teflon homogenizer, and filtered through nylon mesh in a volume of 20 ml RPMI 1640 medium (GIBCO) supplemented with 7.5% fetal calf serum (FCS) (Kansas City Biologicals). All tissue, cells, and media were handled aseptically and kept on ice. The cell suspension was centrifuged at 300 $\times$ g for 5 min, and the pellet was resuspended and washed once with medium. Isopycnic sedimentation of cells on Ficoll-paque[®] (Pharmacia Fine Products) was achieved by centrifugation at 400 $\times$ g for 30 min at 20 C; lymphoid cells at the interface were removed by aspiration and washed twice.

Exposure and Stimulation with Mitogen. Exposure of cells to varying concentrations of metabolites or reagents was carried out by preincubation at 37 C in phosphate-buffered saline (pH 7.2). Thoroughly washed cells were plated at a density of 2×10^5 cells per well in 96-well, flat bottom microtiter plates (Bellco) containing 0.2 ml RPMI 1640 medium supplemented to final concentration with

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7.5% FCS, 2 mM L-glutamine, 100 μ /ml penicillin, 100 μ g/ml streptomycin (as a mixed supplement from Microbiological Associates), and 5×10^{-5} M 2-mercaptoethanol (Sigma). Plates were incubated at 37 C in a humidified atmosphere consisting of 5% CO₂ 95% air. Optimal blastogenic response to 5 μ g/ml phytohemagglutinin (PHA) (Burroughs Wellcome) occurred on day 3 of culture. Synthesis of DNA was assayed by adding 0.2 μ Ci of [³H]thymidine (specific activity, 6.7 Ci/mmol; New England Nuclear) per culture 6 hr prior to harvest. Samples were collected on filter paper, dried, and counted by conventional liquid scintillation methods. Cells were examined by phase contrast microscopy, and an agglutination index was scored on a scale from 0 to 4+ (44). 0 indicated no evidence of aggregation; 1+ represented the presence of clusters containing less than 50 cells interspersed with individual cells and was encountered in cultures containing no mitogen; 2+ to 4+ represented progressively larger aggregates containing blast cells with fewer individual cells.

Isolation and Characterization of Rat Brain Tubulin. Brain tubulin was isolated from male Fischer 344 rats by the cycle procedure (6, 7, 25, 47). Tubulin prepared in this manner was stored at -80 C and used within 2 weeks. Temperature-dependent polymerization at 37 C was measured turbidometrically at 350 nm as described elsewhere (12, 13). Tubulin polymerization experiments were conducted using 20-mM phosphate buffer (pH 6.92). Measurement of polymerization under conditions producing greater than 80% inhibition were not reported due to partial denaturation of the tubulin. Denaturation was determined to be significant when turbidity (log absorbance plotted versus the log of the wavelength) failed to fulfill the criteria of the Raleigh-Gans approximation for long thin rods (4, 12, 54). Decay of TCBA was determined by using [³H]colchicine (1 Ci/mmol) and gel-filtration as described elsewhere (25) except substituting 20-mM phosphate buffer. The pH of reagent and assay solutions was maintained at 6.92 ± 0.02 . In assessing the effects of various treatments on tubulin polymerization or TCBA decay, replicate experiments were conducted on different batches of tubulin and on different days. Results were analyzed by 2-way analysis of variance using a randomized complete block design, and mean differences were evaluated for significance at the $p = 0.05$ level using Duncan's multiple range test (50).

Spectral Analyses. The stability of p-BQ and HQ in the presence or absence of protein was compared by measuring changes in their absorption maxima (248 and 288 nm, respectively) with time. Spectral measurements were conducted at 37 C using a Cary model 219 scanning spectrophotometer. All readings were blanked against buffer or the addition of protein.

Colchicine, CT, L-cysteine (CYS), dithiothreitol (DTT), DL-lysine, L-serine, GTP, NEM, and tyrosinase (EC. 1.14.18.1) were purchased from Sigma. HQ, p-BQ, and 1,2,4-benzenetriol (1,2,4-BT) were obtained from Aldrich.

RESULTS

Effect of HQ and BQ on kctin-Induced Activation of Lymphocytes in Culture. Hydroquinone modified PHA-stimulated DNA synthesis in lymphocytes in a biphasic manner (Table 1) as assessed by [³H]thymidine uptake. Concentrations of less than 1 μ M enhanced PHA stimulation while concentrations of greater than 1 μ M suppressed mitogen response. Enhancement was optimal at approximately 1×10^{-7} M HQ and became progressively smaller at lower concentrations where it

TABLE I
Effect of Hydroquinone, *p*-Benzoquinone or Catechol on PHA Stimulation of Rat Spleen Lymphocytes

Concentration ^a ($\times 10^{-7}$ M)	Hydroquinone			<i>p</i> -Benzoquinone			Catechol		
	[³ H]TdR ^b dpm $\times 10^{-3}$	% ^c	Agglutination Index	[³ H]TdR dpm $\times 10^{-3}$	%	Agglutination Index	[³ H]TdR dpm $\times 10^{-3}$	%	Agglutination Index
0	8.2	-	4+	32.3	-	4+	32.3	-	4+
4	16.8	+105	4+	24.2	-25	4+	38.1	+18	4+
6	15.5	+89	4+	23.2	-28	4+	36.9	+14	4+
8	12.7	+55	4+	12.4	-62	3+	33.2	+3	4+
10	11.1	+35	4+	14.4	-55	3+	33.9	+5	4+
20	2.6	-68	3+	0.5	-98	2+	34.6	+7	4+
40	0.4	-95	2+	0.2	-99	0	42.3	+31	4+
60	0.2	-98	1+	0.2	-99	0	38.5	+19	4+
80	0.2	-98	0	0.1	-100	0	25.6	-21	4+
100	0.2	-98	0	0.2	-99	0	32.8	+2	4+

^aCells were preincubated with metabolite at various concentrations for 14 hr.

^bAt 72 hr after addition of stimulus (5 μ g/ml PHA). DNA synthesis was assayed by the measurement of [³H]thymidine uptake. Values represent the mean of triplicate cultures. SD of all mean values $> 1,000$ dpm varied less than 10%. Stimulation by mitogen resulted in a 50- to 100-fold increase in the incorporation of label above background.

^cPercentage difference (caused by treatment) from control.

*Cells stimulated more than PHA-stimulated control

hydroquinone inhibited Catechol. The degree of stimulation followed the pattern of penetrance or NE dependence of culture

Treatment
Hydroquinone

Hydroquinone

Hydroquinone

NEM

NEM

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TABLE 2
Effect of Cysteine on Inhibition of Lymphocyte
Stimulation by Hydroquinone or N-ethylmaleimide*

Treatment	Concentration (M)	Concentration CYS (M)	% Control dpm [3 H]TdR	Agglutination Index
Hydroquinone	1×10^{-6}	None	103	4+
	1×10^{-5}	None	1	0
	1×10^{-4}	None	0	0
Hydroquinone	1×10^{-6}	1×10^{-4}	98	4+
	1×10^{-5}	1×10^{-4}	98	4+
	1×10^{-4}	1×10^{-4}	41	3+
Hydroquinone	1×10^{-6}	1×10^{-3}	103	4+
	1×10^{-5}	1×10^{-3}	115	4+
	1×10^{-4}	1×10^{-3}	92	4+
NEM	1×10^{-6}	None	31	3+
	1×10^{-5}	None	1	0
	1×10^{-4}	None	0	0
NEM	1×10^{-6}	1×10^{-4}	83	4+
	1×10^{-5}	1×10^{-4}	90	4+
	1×10^{-4}	1×10^{-4}	1	0
NEM	1×10^{-6}	1×10^{-3}	57	4+
	1×10^{-5}	1×10^{-3}	80	4+
	1×10^{-4}	1×10^{-3}	77	4+

*Cells were preincubated for 30 min with HQ or NEM in the presence or absence of CYS prior to stimulation with PHA as given in Table 1. Values are the means of triplicate cultures. SD did not vary more than 10% for any mean > 1,000 dpm. In the HQ experiment, [3 H]TdR incorporation in control, PHA-stimulated cultures equalled 72,500 dpm compared with 2,000 in unstimulated cultures. For NEM, control PHA stimulation was 65,500 dpm compared with 1,800 in unstimulated cultures.

approached control values at 1×10^{-8} M. Cell agglutination was also inhibited at HQ concentrations which suppressed blastogenic response. *p*-BQ produced a similar inhibition of blastogenesis, although it was approximately twice as potent as HQ. Catechol had no significant effect on either mitogen response or cell agglutination.

The data presented in Table 1 are the results of a single experiment. Enhancement of the PHA response was repeatedly observed over the same concentration range (1×10^{-9} to 1×10^{-6} M) for HQ and *p*-BQ, although the degree of enhancement varied from one experiment to another. No correlation was observed between the degree of enhancement and the amount of [3 H]TdR uptake in control PHA-stimulated cultures. For example, in other experiments, pretreatment with HQ resulted in significant enhancement of blastogenesis with [3 H]TdR uptake in control cultures ranging between 58,000 and 73,000 dpm.

Concurrent inhibition of cell blastogenesis and agglutination was also observed following pretreatment with NEM (Table 2). Pretreatment with DTNB, a poorly penetrating SH reagent (1, 11), failed to inhibit either agglutination or PHA mitogen response. The addition of an SH compound, CYS, to the incubation with either HQ or NEM protected against the inhibitory effects of both agents in a concentration-dependent manner (Table 2). Concomitant treatment of HQ- or NEM-treated cultures with 1×10^{-3} M lysine or serine offered no protection against inhibition of

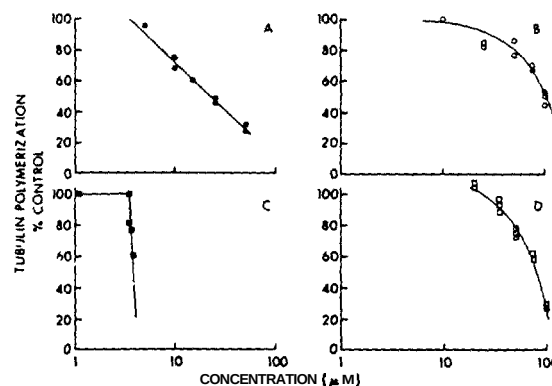


Fig. 1. Log concentration-inhibition curves for (A) p-BQ, (B) HQ, (C) NEM, or (D) 1,2,4-BT on purified tubulin polymerization *in vitro*. Inhibition of polymerization was calculated as the percent of control for incubation with different concentrations of inhibitors.

cell response; although DTT, another SH compound, did provide concentration dependent protection. These findings indicate that HQ suppressed blastogenic response *via* interaction with intracellular SH sites.

Effect of Benzene Metabolites on Tubulin Polymerization. Tubulin polymerization was examined in the presence of HQ, p-BQ, 1,2,4-BT or NEM. 1,2,4-BT is a putative oxidation product of CT and has been shown to autoxidize in a reaction analogous to HQ (26). Polymerization was inhibited by p-BQ, HQ, 1,2,4-BT, or NEM in a concentration-dependent manner (Fig. 1). The linearity of a semi-logarithmic plot of inhibition compared with the concentration of p-BQ or NEM is characteristic of a first order reaction and is suggestive of a unimolecular process in which p-BQ and NEM react directly to inhibit tubulin polymerization. Minimally effective concentrations of p-BQ and NEM were the same, although differences in the slope of the lines may reflect increased affinity for some SH sites by NEM relative to p-BQ. These results were contrasted by the nonlinearity exhibited for HQ and 1,2,4-BT inhibition of polymerization when plotted in the same manner. Linearity on a log-log plot would indicate that inhibition by these compounds could be characterized as either second order or the sum of the products of 2 first order processes. A log-log plot of the data presented in Figures 1 B or D failed to yield a linear transformation, indicating that the kinetics of inhibition of tubulin polymerization by HQ and 1,2,4-BT are of a higher order of complexity. Catechol had no

TABLE 3
Effect of Tyrosinase on Catechol Inhibition of Tubulin Polymerization

Tubulin Polymerization (% Control)		
Catechol (1×10^{-5} M)	Tyrosinase (10 μ g/ml)	Catechol + Tyrosinase
104 ± 1.9^a	94.7 ± 0.9^a	8 ± 2^b

^aMean $\pm$ SD of 3 separate determinations.

^bMean $\pm$ SD of 4 separate determinations.

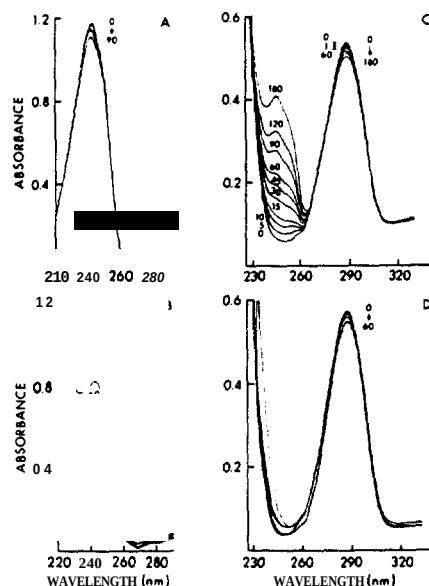


Fig. 2. Effect of protein on the autoxidation of HQ and the disappearance of p-BQ under physiologic conditions. (A) Decomposition of p-BQ (0.05 mM absorption maximum = 248 nm) in 20-mM phosphate buffer (37°C, pH 6.92). (B) Disappearance of p-BQ following addition of bovine serum albumin (2 mg/ml). (C) Autoxidation of HQ (0.2 mM, absorption maximum = 288 nm) and the appearance of p-BQ. (D) Autoxidation of HQ in the presence of albumin. Numbers indicate the scan time (min) after the addition of p-BQ or HQ.

effect on tubulin polymerization at concentrations of 5×10^{-4} M, a finding which was consistent with its failure to autoxidize appreciably under physiologic conditions (26). However, 1,2,4-BT, a putative enzymatic oxidation product of CT, was approximately twice as potent as HQ in inhibiting tubulin polymerization. 1,2,4-BT has been demonstrated to autoxidize more rapidly than HQ (26).

In order to further assess the role of quinones in the inhibition of tubulin polymerization, CT was added to tubulin in the presence of tyrosinase (10 μ g/ml). Tyrosinase converts CT to the o-quinone directly via 2 electron transfers, thus, forming no reactive intermediates in the reaction (34). Theo-quinones are reported to be extremely electron-deficient species capable of reacting with external nucleophiles such as SH, amino or phenolic hydroxyl groups and have been shown to react with SH groups in preference to other intramolecular reactions (48). In the tyrosinase-catechol system as little as 1×10^{-5} M CT resulted in complete loss of tubulin polymerization and partial denaturation of the protein (Table 3). Tyrosinase alone had no effect on tubulin polymerization. Consistent with its greater SH reactivity, o-BQ appears to be a more potent inhibitor of tubulin polymerization than does p-BQ. These findings suggest a direct benzoquinones-tubulin interaction similar in nature to that observed with NEM.

Stability of p-BQ and HQ in the Absence and Presence of Protein. Spectral scans of p-BQ and HQ in the absence or presence of albumin revealed that the rate of disappearance of absorption maxima for p-BQ normally seen with time was greatly accelerated by the addition of protein (Figs. 2 A and B). The rate of disappearance

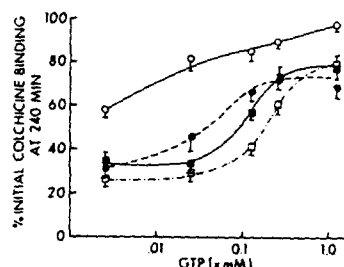


Fig. 3. Concentration-response curves of TCBA decay at varying concentrations of added GTP. Purified tubulin preparations containing 2×10^{-6} M [3 H]colchicine ($0.1 \mu\text{Ci}$) were incubated at 37°C for 240 min in the presence of varying concentrations of GTP (abscissa) with or without (—○—) the addition of 2×10^{-4} M *p*-BQ (—●—), NEM (—■—), or 1,2,4-BT (—□—). Initial TCBA was determined by extrapolation of the decay curve for untreated tubulin preparations to time zero. Values represent the mean $\pm$ SEM for 3 separate experiments.

of absorption maxima for HQ was slightly enhanced by the addition of protein, but the most striking feature of these profiles was the absence of the appearance of an absorption maxima for *p*-BQ (Figs. 2 C and D). These findings are consistent with a direct attack by *p*-BQ on the protein but, taken alone, do not rule out a role for the *p*-semiquinone radical in the reaction.

Measurement of TCBA Decay. The decay of TCBA coincides with the irreversible loss of tubulin polymerization and denaturation of the protein (28, 60). Decay of TCBA can be attenuated by the addition of DTT, GTP or other agents, eg., glycerol, which stabilize free thiol groups on the tubulin molecule (33, 38). We have previously used TCBA decay as a measure of the specificity of interaction with SH groups or GTP-associated binding to tubulin: TCBA decay is accelerated in the presence of HQ and is prevented in a concentration-dependent manner by the addition of GTP (25). GTP had a similar protective effect on acceleration of TCBA decay by *p*-BQ, 1,2,4-BT or NEM (Fig. 3); however, complete protection against the acceleration of TCBA decay was not observed. The SH specificity of the interaction of *p*-BQ with tubulin is illustrated in Figure 4. Dithiothreitol, but not lysine or serine, afforded complete protection against the effects of either *p*-BQ or NEM on TCBA decay. Although differences in TCBA between control and DTT-treated incubations were not significant, the tendency of DTT to retard TCBA decay was consistent with a role for free SH groups in the maintenance of microtubule stability. We have shown the acceleration of TCBA decay by HQ to be oxygen-dependent (25). Incubation under nominally anaerobic conditions (Fig. 5) provided significant protection against 1,2,4-BT but not against *p*-BQ or NEM. This finding is thought to reflect the requirement for autooxidation of HQ or 1,2,4-BT in the expression of toxicity. Conversely, the interaction of *p*-BQ or NEM with tubulin does not appear to be an oxidative process.

DISCUSSION

A number of observations support the hypothesis, first put forth by Edelman (10), that regulation of cell division is modulated by microtubule interaction with cell surface structures (36, 37). Microtubule-disrupting agents, including SH reagents, must permeate the cell in order to inhibit functions requiring conformational change

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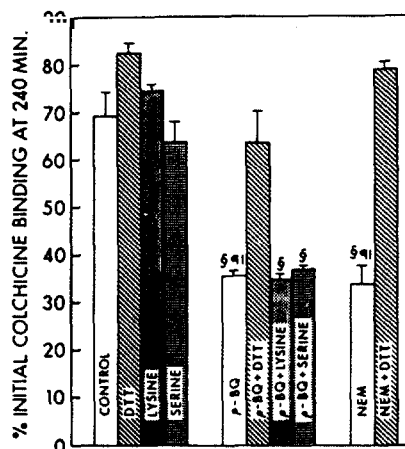


Fig. 4. Effect of various compounds on the acceleration of TCBA decay by p-BQ or NEM. Incubation conditions were the same as those described in the legend to Figure 3 except that the concentration of GTP (2.5×10^{-5} M) was constant throughout. Concentrations of DTT, lysine, serine, p-BQ, and NEM were all 1×10^{-4} M. Values represent the mean $\pm$ SEM for 3 separate experiments. (§) Significantly different from control ($P < 0.05$). (¶) Significantly different from DTT treated.

(2, 10, 11, 36, 37, 55). Although there is not uniform agreement on the importance of direct cell-cell communication in the initiation of cell division, agglutination has been reported to be a requisite for blastogenesis and represents one of the earliest events associated with initiation of cell division (32, 57). Studies have repeatedly demonstrated that inhibition of blastogenesis by microtubule-disrupting agents or SH reagents does not involve alterations in lectin-binding to surface receptors (3, 9, 16, 45), alterations in GSH concentrations (9, 30), or interference with energy production within the cell (8, 30, 35). Our findings provide additional confirmation that microtubule-disruptive agents inhibit cell agglutination and simultaneously interfere with density-dependent stimulation of cell growth (3, 36, 37, 44). In addition, these findings indicate a) that suppression of lymphocyte activation by polyhydroxy-metabolites of benzene is mediated through inhibition of microtubule

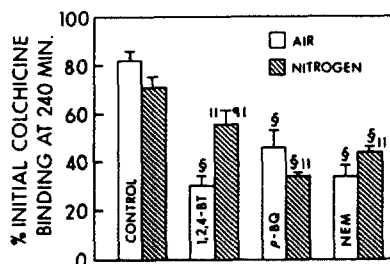


Fig. 5. Effect of anaerobic conditions on acceleration of TCBA decay by 1,2,4-BT, p-BQ, or NEM. Aerobic conditions were the same as those described in the legend to Figure 4. Anaerobic incubations were conducted using degassed solutions saturated with N_2 . Values represent the mean $\pm$ SEM for 3 separate experiments. (§) Significantly different from control. (¶) Significantly different from same incubation in air. (¶) Significantly different from anaerobic control.

assembly, b) that both processes are SH-dependent, and c) that benzoquinones are the most likely molecular species responsible for direct interaction at SH-dependent sites on microtubules. p-Benzoquinone, the autoxidation product of HQ, is more potent in both systems: the metabolites suppress both lymphocyte response and microtubule assembly at the same concentrations. N-ethylmaleimide, an SH-alkylating agent, mimicked the effects of benzene metabolites, and sulfhydryl compounds, provided protection against the effects of both NEM and the metabolites on lymphocyte activation and microtubule function. Pretreatment with a variety of compounds demonstrated the dependence of these processes on specific intracellular SH groups. Analysis of kinetic and spectral data suggests a molecular mechanism in which microtubule-associated SH groups demonstrate particularly reactive nucleophilicity toward quinones or semiquinones.

Concentrations of HQ or p-BQ (1×10^{-6} to 1×10^{-5} M) which suppress lymphocyte activation in culture are the same as those producing inhibition of tubulin polymerization *in vitro*. Inhibition of lectin-stimulated blastogenesis coincides with the loss of cell agglutination. Only a small portion of the decrease in [3 H]thymidine uptake observed following 14-hr preincubation with HQ or p-BQ can be accounted for by cell loss. Furthermore, we have reported other experiments in which incubation for 30 min with the same concentrations of HQ and p-BQ results in a complete loss of mitogenic activity in the absence of cell loss or a decrease in energy production (44). Complete protection against the suppressive action of HQ or p-BQ on lymphocyte activation is afforded by CYS but not lysine or serine. NEM inhibits blastogenesis and agglutination at the same concentrations as p-BQ which is protected against by CYS, whereas DTNB has no effect on either lymphocyte activation or agglutination. These observations are indicative of an interaction with intracellular SH groups critical to lectin-stimulated lymphocyte activation. Analogous observations with respect to SH specificity have been presented for the effects of p-BQ on tubulin stability *in vitro*.

We have recently reported that HQ covalently binds to tubulin (25). The ratio of HQ:tubulin concentration which is attended by inhibition of polymerization is consistent with an interaction involving a very small number of binding sites. Observations presented in this paper implicate p-BQ as the ultimate toxic species in a reaction scheme in which HQ is autoxidized to p-BQ, although due to the circumstantial nature of the evidence one cannot entirely rule out the possibility that the p-benzosemiquinone intermediate is active as well. In either case proposals for the chemical reaction scheme between p-BQ or p-benzosemiquinone and tubulin are consistent with conjugate addition in which a highly polarized unsaturated carbon-carbon bond is subject to attack by a reactive thiol group acting as a nucleophile (39, 49). Identical models for thiol alkylation are provided by other inhibitors of tubulin polymerization, namely NEM and cytochalasin A (20, 29, 30). The denaturation of tubulin by CT following the addition of tyrosinase suggests a greater reactivity of the o-BQ. This observation is supported by studies of the oxidation products of 6-hydroxydopamine in which the o-quinones possessed greater SH reactivity than did the p-quinones, based on their ability to inhibit SH-dependent enzymes. The demonstrated reactivity of p-BQ with tubulin suggests that tubulin may represent a more sensitive indicator of SH reactivity than do enzymes such as CT-o-methyl transferase or calf thymus DNA polymerase (5, 15).

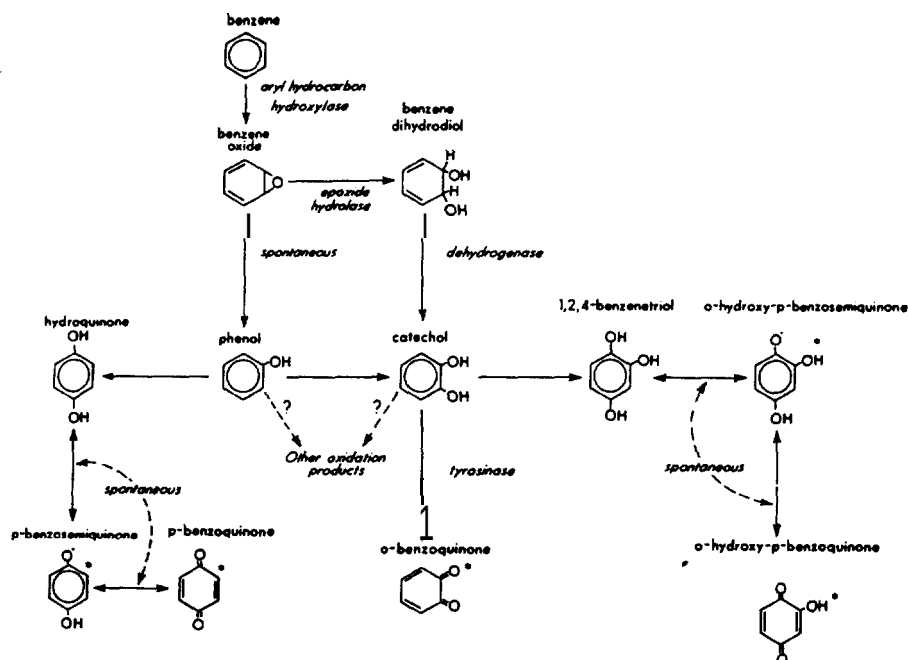


Fig. 6. Schematic diagram of primary benzene metabolism resulting in the production of potentially lymphotoxic metabolites. (*) Putative or demonstrated alkylating activity toward intracellular nucleophilic groups.

Depending on the endpoint used to measure toxicity, benzene metabolites may present with varying orders of potency. Hydroquinone is a more potent inhibitor of lymphocyte activation than is CT *in vitro* (26, 44) and *in vivo* (59). On the other hand, CT is more potent at producing sister chromatid exchange in human lymphocytes in culture (40). Such discrepancies could arise from differences in the oxidation products of HQ and CT. We propose a reaction scheme in which HQ and CT can proceed along parallel or distinct oxidative pathways (Fig. 6). Hydroquinone spontaneously oxidizes to form p-benzosemiquinone and p-BQ. Catechol does not undergo appreciable autooxidation under physiologic conditions, but it could serve as a substrate for a variety of enzymatic reactions. Catechol can be oxidized to 1,2,4-BT which undergoes a similar autooxidation reaction to HQ, presumably forming the 2-hydroxy-analogue of p-BQ. Alternatively, CT may be oxidized by tyrosinase or a variety of oxidases to form potentially reactive products such as phenoxy-radicals or o-quinones which would be expected to react indiscriminately with a variety of nucleophilic groups on protein or DNA in addition to SH groups. The relatively weak alkylating ability of the p-quinones could result in specificity for particularly nucleophilic SH groups within the cell and account for their ability to interfere with cytoskeletal function in the absence of cytotoxicity. The observation that quinone metabolites interfere with normal cytoskeletal function suggests a mechanism to explain bone marrow and immune suppression associated with chronic benzene exposure.

ACKNOWLEDGMENT

The authors thank Dr. Ronald P. Mason of the National Institute of Environmental Health Sciences and Mr. Robert O. Beauchamp for helpful discussions.

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