Arch. Toxicol. 45. 297–305 (1980)

Archives of TOXICOLOGY

Effects of the Principal Hydroxy-metabolites of Benzene on Microtubule Polymerization

Richard D. Irons and Douglas A. Neptun

Chemical Industry Institute of Toxicology, Research Triangle Park, North Carolina, NC 27709, USA

Abstract. The principal hydroxy-metabolites of benzene – phenol, catechol and hydroquinone – possess characteristics and produce toxicity similar to those reported for certain inhibitors of microtubule polymerization. In this study we examined the effects of phenol, catechol and hydroquinone on purified microtubule polymerization and the decay of tubulin-colchicine binding activity. Hydroquinone. but not catechol or phenol, inhibited microtubule polymerization and accelerated the decay of tubnlin-colchicine binding activity. The latter effect was shown to be dependent on the concentration of GTP. Hydroquinone did not directly complex with GTP or ATP but bound to the high molecular weight fraction of tubulin. Concentration ratios of hydroquinone to tubulin resulting in altered activity were low, suggesting a specific interaction, presumably at the tubulin-GTP binding site. The acceleration of tubulin-colchicine binding activity decay was completely prevented under anaerobic conditions, indicative of an oxidative mechanism. These studies suggest that hydroquinone, which auto-oxidizes, may interfere with microtubule function, nucleotide binding or both and that this mechanism may be involved in eliciting the wide range of cytoskeletal-related abnormalities observed in cells exposed to benzene in vivo or its metabolites in vitro.

Key words: Benzene – Hydroquinone – Microtubules – GTP – Auto-oxidation.

Introduction

Microtubules, which are polymers of the tubulin molecule, are known to participate in several structurally related processes within eukaryotic cells. Microtubule integrity is a requirement for spindle formation during cell division, maintenance of cell shape, intracellular organelle movement and secretion of cellular products (Bryan 1974; Lacey et al. 1968: Katz, 1972). Microtubules,

0340-5761/80/0045/0297/$ 01.80

microfilaments and their associated proteins are also involved in the regulation of cell surface receptor mobility and cell growth (Edelmann 1976; Mc-Clain and Edelman 1980).

A number of different compounds are known to inhibit tubulin polymerization or to accelerate tubulin depolymerization including: colchicine. vinblastine. diphenylhydantoin and selected sulfhydryl reagents (Wilson et al. 1973: MacKinney et al. 1978; Himes and Himes 1980: Mellon and Rebhun 1976). Exposure of cells to these agents in vivo or in culture results in metaphase arrest. uneven nuclear division and chromosomal abnormalities (MacKinney et al. 1978; Himes and Hirnes 1980; Deysson 1975). Additional compounds which block division. bind to tubulin or inhibit tubulin polymerization possess structures similar to catechol or hydroquinone, the principal dihydroxymetabolites of benzene (Traganos et al. 1980; Misurni et al. 1979). Administration of benzene to rats or rabbits results in cycle specific changes in the kinetics of proliferating cells in bone marrow indicative of G_2/M phase arrest (Irons et al. 1979; Irons, 1980; Muirhead, et al. 1980), and a number of studies have reported abnormalities of cell division consistent with mitotic arrest following benzene exposure (16). Phenol, catechol and hydroquinone have been identified as mitotic inhibitors of cells in culture as well (Parmentier and Dustin 1953).

It is generally accepted that benzene is not directly toxic but that toxicity is dependent on the metabolism of the compound to a reactive species (Laskin and Goldstein 1977). Recent studies in our laboratory indicate that polyhydroxy-metabolites of benzene – hydroquinone. catechol. benzoquinone and 1.2.4-benzenetriol – suppress mitogen stimulated lymphocyte response in culture at non-cytotoxic concentrations (Wierda et al. 1980), and under physiologic conditions both hydroquinonc and 1,2,4-benzenetriol are subject to auto-oxidation (Greenlce and Bus 1980).

In this paper we report the inhibition of microtubulin polymerization and the accelerated decay of tubulin-colchicine binding activity (TCBA) by hydroquinone in vitro. Phenol and catechol were also investigated but had no effect on these processes. Hydroquinone does not alter the affinity of colchicine binding to tubulin but apparently competes for sulfhydryl dependent GTP-binding sitcs on the protein. This is prevented under anaerobic conditions, indicative of an oxidative process. Similar effectson GTP binding to tubulin have been reportcd for selected sulfhydryl reagents which preferentially bind to -SH groups at the GTP-binding site at higher rates than to other -SH groups on the protein molecule (Mann et al. 1978). These findings offer a possible explanation for alterations in cell growth or differentiation resulting from exposure to benzene or its metabolites through disruption of rnicrotubulcs. interference with GTP other nuclrotidc dependent processes or both.

Materials and Methods

Chemicals. [^{14}C]hydroquinone. [^{14}C]catechol and [^{14}C]phenol (37.9 Ci mol) were obtained from Midwest Research Institute and [^{3}H]colchicine (23.2 Ci mmol) Irom New England Nuclear. Colchicine and catechol were obtained Irom Sigma and hydroquinone and phenol from Aldrich

Effects of Benz

Isolation of
(≥ 250 g) as des
anesulfonic acic
GTPJ. The pH

Measurement of
measured turbi
spectrophotome
corrected for bu
merhod (Gorn
expcrimcnts.

Assay of Colch
assayed by gel
addition of GTP
3.7×10^{10} beeq
binding was ass

Electrophoresis.
out using the
amino-mcrhane
paper chromato
and Isherwood

Results

Hydroquino
polymerizati
concentratio
(IC_{50}) was
Colchicine it
affinity of in

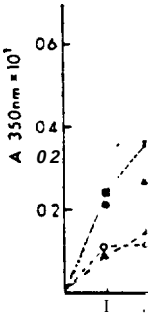

Fig. 1. Effect
hydroquinone
butter. mainta
Samples were
absorbance me
were correcte

Isolation of Rat Brain Microtubulin. Rat brain tubulin was isolatsd from male Fischer-344 rats (≥ 250 g) as dcscribcd (Borisy et al. 1974, 1975) using PEG buffer [0.1 M piperazine-N,N'-bis(2-ethanesulfonic acid): 1 mM EGTA (ethylene glycol bis(β-aminoethylether)tetraacetic acid: 2.5 mM GTP]. The pH of all incubations was monitored and maintained at 6.94 ± 0.02.

Measurement of Tubulin Polymerization. The temperature dependent polymerization of tubulin was measured turbidometrically (Gaskin et al. 1975) at 350 nm using a Gilford Stasar model III spectrophotometer equipped with a temperature controlled flow cell. All measurements werc corrected for buffer and reagent absorbance. Protein concentrations werc determined by the biuret method (Gornall et al. 1949) and adjusted with buifer to 1.7 mg/ml for polymerization experiments.

Assay of Colchicine-Tubulin Binding Decay. Colchicine-tubulin binding and TCBX decay were assayed by gel filtration as described (Wilson et al. 1974) using the isolation buffer without the addition of GTP (PIPES). For determination of colchicine binding. 0.1 μCi of [^{3}H]colchicine (1 Ci = 3.7×10^{10} becquerals) was added in varying concentrations to the incubations. [^{14}C]hydroquinone binding was assayed using the same method (0.1 μCi/assay).

Electrophoresis. Sodium dodscyl sulfate (SDS) polyacrylamide disc gel electrophoresis was carried out using the method of Shapiro et al. (1967) modified using TRIS [tris(hydroxymethyl) amino-methane)] buffer. 0.4 M, pH 8.8. ^{14}C-labelled metabolite-nucleotide binding was assayed by paper chromatography as dcscribcd (MacKinney et al. 1978) and nucleotides identified using Hanes and Isherwood reagent (Zweig et al. 1972).

Results

Hydroquinone, but not catechol or phcnol. inhihited purified microtubuiin polymerization in vitro in a concentration dependent manner (Fig. 1); the concentration of hydroquinone producing a 50% inhibiton of polymerization (IC_{50}) was approximately 10-fold greater than that of colchicine (Fig. 2). Colchicine is thought to inhibit polymerization by binding to or altering the affinity of interaction sites between tubulin molecules (Wilson and Meza 1973).

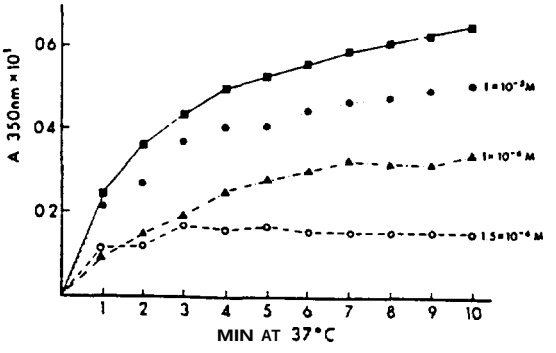

Fig. 1. Effect of hydroquinone on the polymerization of microtubules. Variable amounts of hydroquinone (0, 1, 10, or 15 μl) werc added to 1.7 mg of purified rat brain tubulin in 1 ml of PEG buffer. maintained at 0° C. to give final concentrations of hydroquinone as indicated (● ▲ ○). Samples were introduced into the spectrophotometer flow ccll. equilibrated at 37° C in 20 s. and absorbance monitored for 10 min. Carrier buffer alone was added to control samples (■). Readings werc corrected lor reagent-buffer absorbance

To determine whether hydroquinone interfered with tubulin polymerization in a similar manner. the effects of hydroquinone on colchicine-tubulin binding were examined (Fig. 3). The binding constant (K_A) for colchicine-tubulin was found to be 1.54×10^{7} l/mol which is in good agreement with that (2×10^{6} l/mol) reported for chick embryo brain tubulin (Wilson et ai. 1974). Hydroquinone had no effect on either the affinity or the number of colchicine binding sites on tubulin.

TCBA was found to decay in a first-order manner as previously described (Wilson et al. 1974; Weisenberg et al. 1968). Addition of excess GTP. which is normally bound to the tubulin molecule in a molar ratio of 1:1 (Wilson et al. 1974), stabilized TCBA; however, hydroquinone markedly accelerated the decay of TCBA (Fig. 4). Hydroquinone interfered with the GTP-dependent stabilization of TCBA in a dose dependent manner (Fig. 5). Equimolar

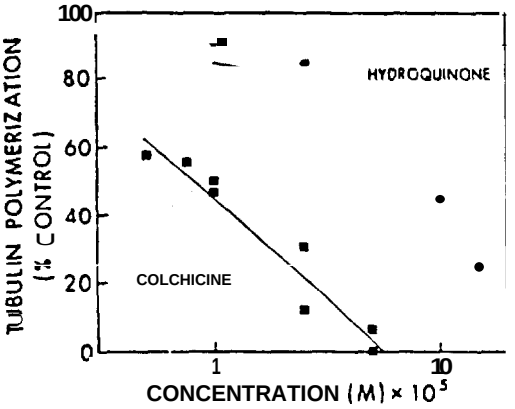

Fig. 2. Concenrration-response curves for colchicine and hydroquinone inhibition of microtubule polymerization. Inhibition of polymerization was calculated as % of control (ordinate) after 10 min incubation at 37° C for different concentrations (abscissa) of colchicine (■) or hydroquinone (●)

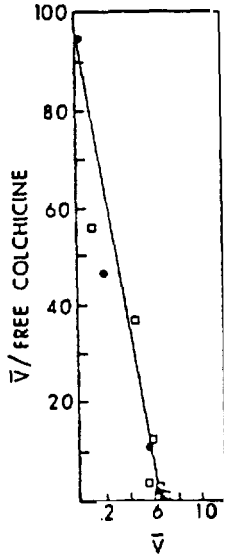

Fig. 3. Scatchard plot of colchicine binding to purified rat tubulin. The binding of [^{3}H]colchicine to tubulin (1 mg/ml) 120 min after incubation at 37° C alone (●) or in the presence of 2×10^{-4} M hydroquinone (□) was examined for various concentrations of colchicine. The molar ratio of hydroquinone to tubulin was 23:1

concentrations c
relative to contr
lowest concentr;
of TCBA incre
increase of [^{14}C]
Incubation unde
TCBA decay (Fi
tubulin and the
[^{14}C]hydroquino
ATP in PEG b
chromatograhpy
radioactivity app
that of the nuc
catcchol.

hlasimurn m
parentheses) we

Fig. 4. Effect of hy
TCBA. Purified tu
M [^{3}H]colchicine (
the presence of 2.5
of GTP (●) or in t
2×10^{-4} M hydroqu

Fig. 5. Concentratior
decay at varying con
Purified tubulin prep
M [^{3}H]colchicine (0.
37° C for 360 min in
concentrations of GT
(●) the addition of
(○), catechol (■) or
mean ± SE for 3 (t
experiments

erization in a
binding were
n was found
x 10^6 l/mol)
.quinone had
ling sites on

ly described
TP. which is
Wilson et al.
elerated the
P-dependent
. Equimolar

ponse curves
quinone

of
lated as %
r 10 min
ifferent
of colchicinc

rot tubulin.
min after
< 10^{-4} M
ions of
was 23:1

concentrations of GTP and hydroquinone resulted in a 50% decrease in **TCBA.** relative to control preparations. Phenol and catechol were effective only at the lowest concentration of GTP. The effect of hydroquinone on GTP stabilization of TCBA increased with time **(Fig.** 4) which correlated with the first-order increase of [^{14}C]hydroquinone binding to tubulin with time (data not shown). Incubation under nitrogen completely inhibited the effect **of** hydroquinone on TCBA decay (Fig. **6).** To distinguish between a direct effect of hydroquinone on tubulin and the formation of **a** hydroquinone-nucleotide complex. **0.4** mM [^{14}C]**hydroquinone (2** μCi) was incubated for **2** h with 7.6 mM **GTP** or 6.9 mM **ATP** in PEG buffer at **37°C.** Separation of respective fractions by paper chromatograhpy revealed no ^{14}C-label migrating with GTP or **ATP,** all radioactivity appearing as a single peak with a mobility approximately one half that of the nucleotides. Identical results were obtained with phenol and catechol.

Maximum molar ratios of hydroquinone: tubulin (ratios are given in parentheses) were calculated for concentrations of the benzene metabolite

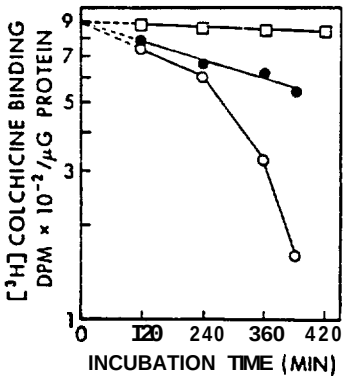

Fig. 4. Effect **of** hydroquinone and **GTP** on TCBA. Purified tubulin containing 2 × 10^{-6} M [^{3}H]**colchicine (0.1** μCi), ***was*** incubated in the prcsencc of ***2.5*** mM ***GTP*** (□), absence of GTP (●) or in the absence of GTP with **2 x** 10^{-4} M hydroquinone (O)

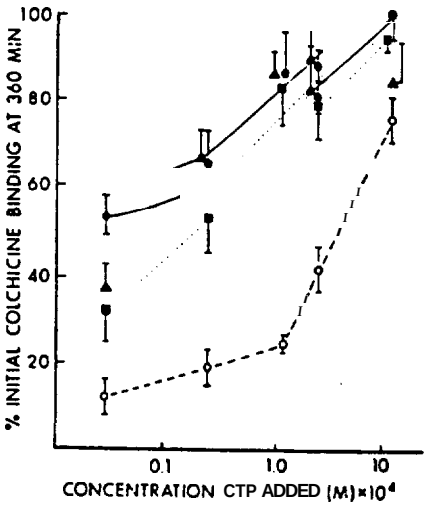

Fig. 5. Concentration-response curves for TCBA decay at varying concentrations of added GTP. Purified tubulin preparations. containing 2 **x** 10^{-6} **M** [^{3}H]colchicine (0.1 μCi), werc incubated **at 37"C for** 360 min in the presence of varying concentrations of GTP (abscissa); with or without ***(O)*** the addition of 2 × 10^{-4} M hydroquinone (O) catcchol (■) or phcnoi (▲). Values present mean ± **SE** for 3 (treated) and 4 (control] experiments

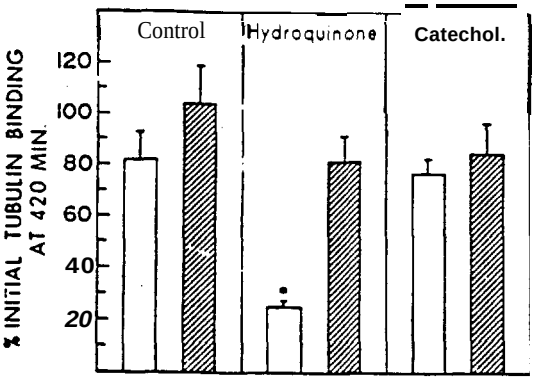

Fig. 6. Effect of anaerobic conditions on hydroquinone acceleration of TCBA decay. Purified tubulin preparations, containing 2×10^{-6} M [^{3}H]colchicine (0.1 μCi) and 1.25×10^{-4} M GTP, were incubated at 3 P C for 360 min with 2×10^{-4} M hydroquinone. catechol or phenol (data not shown) in air (□) or under N_2 (▨). Catechol or phenol had no effect on TCBA either in air or under N_2. Values reprcsenr mean ± for 3 exprcimcnts. * Significantly different from control ($p < 0.006$)

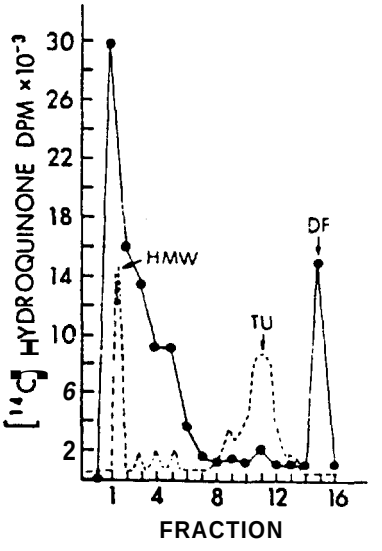

Fig. 7. Electrophoresis of purified rat brain tubulin Rat brain tubulin (300 μg) was incubated for 1 h in the prcsencc of 2×10^{-4} M [^{14}C]hydroquinone (0.5 μCi) and subjected to SDS polyacrylamide disc gel electrophoresis on 8 cm polyacrylamide (7.5%) gels. Counting gels were cui into 0.5 cm slabs. solubilized and radioactivity (●) determined by liquid scintillation. Parallel gels were stained with Coomassie Blue and densitometric profiles (- - -) obtained bv scanning at 525 nm using a Helena densitometer. High molecular weight (HMW) fraction; low molecular weight (TU) fraction: and dye front (DF) are indicutcd.

which produced the following effects: 50% inhibition of tubulin polymerization (0.67: 1); 100% inhibiton of tubulin polymerization (3.3: I); and 50% incrcasc in TCBA decay (2.3: 1). Electrophoresis (Fig. 7) revealed the majority of [^{14}C]hydroquinone-associated radioactivity to be bound to the high molecular weight fraction of tubulin-associated proteins. previously shown to actively incorporate ^{32}P in vivo (Lagnado and Kirasov 1975).

Discussion

A comparison between the effects of benzene and colchicine reveals the following similarities: 1) exposure to high doses in vivo results in a G$_2$/M block in proliferating cells in bone marrow (Muirhead et al. 1980); 2) chromosomal abnormalities indicative of mitotic arrest are observed following in vivo benzene

exposure (Laskin and Goldstein 1977); 3) the principal hydroxymetabolites of benzene are mitotic inhibitors of cells in culture (Parmentier and Dustin 1953); and 4) hydroquinone inhibits purified tubulin polymerization in vitro. Distinguishing benzene from colchicine are: 1) the requirement for metabolism of benzene for toxicity; and 2) differences in the mode of binding and interaction with tubulin between hydroquinone and colchicine.

Hydroquinone does not bind at the colchicine-binding site nor does it alter the affinity of colchicine at the IC_{50} of the former. Hydroquinone binds irreversibly to the high molecular weight fraction of tubulin whereas colchicine binds reversibly to the low molecular weight form (Wilson et al. 1974). The effects reported here for hydroquinone are similar to those reported for the hydantoin compounds (MacKinney et al. 1978); although, hydroquinone appears to be about 10-fold more potent than diphenylhydantion at inhibiting tubulin polymerization. The biochemistry of the TCBA decay phenomenon is not understood but it coincides with an irreversible loss of polymerization (Kuriyama and Sakai 1974) and evidently involves denaturation of the protein (Wilson et al. 1974). Unlike the dissociation of colchicine from tubulin. TCBA decay is irreversible. The integrity of free -SH groups on the tubulin molecule is a functional requisite for stability (Kuriyama and Sakai 1974; Ikeda and Steiner 1978), and agents which stabilize TCBA, such as guanine nucleotides or glycerol. apparently do so through interaction with -SH groups on the protein (Mellon and Rebhun, 1976; Mann et al. 1978). The GTP-binding site on tubulin has been well characterized and involves 2 out of approximately 11 titratable -SH binding sites on the protein (Mann et al. 1978; Iheka and Steiner 1978). These two -SH groups react with sulfhydryl reagents which inhibit tubulin polymerization at rates much higher than other -SH sites on the tubulin molecule (Mann et al. 1978). Colchicine-binding to tubulin is not affected until five or more -SH groups are blocked, and complete inhibition of colchicine-binding is not observed until all -SH groups are blocked (Iheka and Steiner 1978). Hydroquinone does not alter colchicine binding affinity at a hydroquinone: tubulin molar ratio in excess of 20: 1. Hydroquinone does not react directly with GTP but apparently interferes with GTP-tubulin binding in a concentration dependent manner and only under aerobic conditions. The ratio of hydroquinone: tubulin concentration accompanying a loss of tubulin polymerization or acceleration of TCBA decay is consistent with a reaction involving a limited number of binding sites (1–3) and is indicative of a relatively specific interaction with tubulin. This interaction presumably involves the oxidation of -SH groups associated with GTP-tubulin binding.

The dependence of hydroquinone reactivity on oxygen, together with the marked difference in potency of hydroquinone versus catechol or phenol, provides evidence for an oxidative mechanism of action involving tubulin. Hydroquinone spontaneously oxidizes under physiologic conditions whereas phenol and catechol require further enzymatic oxidation in order to form reactive intermediates (Greenlee and Bus 1980; Mason 1979). Assessment of the effects of quinone and related compounds, such a benzoquinone, on purified tubulin integrity and binding offers an in vitro model for the study of mechanisms involved in free radical interaction with macromolecules.

In addition to causing mitotic arrest in culture. hydroquinone inhibits mitogen stimulation of lymphocyte growth at non-cytotoxic concentrations (Wierda et al. 1980), and has been shown to retard tumor growth in mice receiving melanoma transplants (Chavin et al. 1980). Agents which modulate cytoskeletal structure (e.g. inhibitors of microtubule polymerization) inhibit mitogen stimulation of lymphocytes (McClain and Edelman 1980) and alter cell surface receptor mobility (Edelman 1976). Although the functional role of microtubules in the control of these cellular processes remains to be elucidated. the interference of hydroquinone with microtubulin integrity and nucleotide binding may have broad implications for the study of the mechanism of benzene toxicity at the molecular level.

References

Borisy GG, hlarcum. Jhl. Olmstead JB, Murphy DY. Johnson KA (1975) Purification of tubulin and associated high molecular weight proteins from porcine brain and characterization of rnicrotubulc assembly in vitro. Ann NY Acad Sci 253: 107–132

Borisy GG. Olmsted JB. Marcum Jhl. Allen C (1974) Microtubule assembly in vitro. Fed Pruc 33: 167–174

Bryan J (1971) Biochemical properties of microtubules. Fed Proc 33: 152–157

Chavin W, Jelonek EJ Jr. Reed AH. Binder LR (1980) Survival of mice receiving melanoma transplants is promoted by hydroquinone. Science 208 :408–410

Deysson G (1975) Microtubules and antimitotic substances in microtubules and microtubule inhibitors. Borgers hl. DeBrabander M (eds) Elsevier, North Holland New York. pp 427–451

Edelman GN (1976) Surface modulation in cell recognition and cell growth. Science 192:218–219

Gaskin F. Cantor CR. Shelanski ML. (1975) Turbidimetric studies of the in vitro assembly and disassembly of porcine neurotubules. Ann NY Acad Sci 253: 133–146

Gornall AG. Bardaill CJ, David MM (1949) Determination of serum proteins by means of the biuret reaction. J Biol Chcm 177: 751

Greenlee WF. Bus JS (1980) A proposed mechanism for hcnzcnc toxicity: Formation of reactive intermediates from polyphenol metabolites of bcnzcnc. Pharmacologist 22: 389

Himes RH, Himes VB (1980) Inhibition of tubulin assembly by ethylacetyl-acrylate, a sulfhydryl reagent and potential analog of cytochalasin A. Biochem Biophys Acta 621: 338–342

Ikeda Y. Steiner M (1978) Sulfhydryls of platelet tubulin: Their role in polymerization and colchicine binding. Biochemistry 17: 3454–3459

Irons RD (1980) Benzene induced myelotoxicity: Application of flow cytofluorometry for the evaluation of early proliferative changes in bone marrow. Environ Health Perspect (in press)

Irons RD, HeckHd'A, hloorc BJ. Muirhead KA (1979) Effects of short-term benzene administration on bone marrow cell cycle kinetics in the rat. Toxicol Appl Pharmacol 51:399–409

Katz NL (1972) Effects on fros neuromuscular transmission of agents which ncr upon microtubules and microfilaments. Eur J Pharmacol 19: 88–93

Kuriyama R. Sakai H (1974) Role of tubulin-SH groups in polymerization to microtubules. J Biochcm 76: 651–654

Laccy PE. Howell SL. Young DA, Fink CJ (1968) New hypothesis of insulin hormone secretion. Nature 219: 1177–1179

Lagnado JR, Kirasov EP (1975) Studies on the phosphorylation of brain microtubule protein and microtubule-associated lipids in microtubules and microtubule inhibitors. Borgers M. DeBrabander XI (eds) Elsevier, North Holland, New York. pp 127–140

Laskin S. Goldstein BD (1977) Benzene toxicity: A critical evaluation. J. Toxicol. Environ Health (Suppl) 2: 148

MacKinney AA, Vyas R, Powers KJ (1978) Morphologic effect of hydantoin drugs on mitosis and microtubules of cultured human lymphocytes. J Pharmacol Exp Ther 204: 195–202

Mann K. Giesel M, Fasold H. Haase W (1974) Isolation of native microtubules from porcinc brain and characterization of SH groups essential for polymerization at the GTP binding sitcs. FEBS Lett 92: 45–48

Mason RP (1979) Free radical metabolites of foreign compounds and their toxicological significance. Rev Biochcm Toxicol 1: 151–200

McClain DA. Edelman GM (1980) Density-dependent stimulation and inhibition of cell growth by agents that disrupt microtubules. Proc Natl Acad Sci USA 77: 2748–2752

Misumi M, Yamaka H. Akiyama T, Tanaka N (1979) Mechanism of action of alacinomycin A: The interaction with DNA and with tubulin. J Antibiot (Tokyo) 32: 48–52

Mellon MG. Rebhun LI (1976) Sulfhydryls and the in vitro polymerization of tubulin. J Cell Biol 70: 226–238

Muirhead KA, Irons RD, Bruns R, Horan PK (1980) A rabbit bone marrow model system for evaluation of cytotoxicity. J Histochem 28: 526–532

Parmentier R, Dustin P, Jr (1953) On the mechanism of mitotic abnormalities Induced by hydroquinone in animal tissue. Rev Belge Pathol Med Exp 23: 20–30

Shapiro AL. Vinuela E. Maizel JV (1967) Molecular weight estimation of polypeptide chains by electrophoresis in SDS-polyacrylamide gels. Biochem Biophys Res Commun 28: 815–820

Traganos F. Evenson DP. Staiano-Coico L. Darzynkiewicz Z, Melamed M (1980) Action of dihydroxyanthraquinone on cell cycle progression and survival of a variety of cultured mammalian cells. Cancer Res 40: 671–681

Weisenberg RC, Borisy GG, Taylor EW (1968) Colchicine-binding protein of mammalian brain and its relation to microtubules. Biochemistry 7: 4466–4470

Wierda. D. Greenlee WF. Irons RD (1980) Immunotoxicity of benzene metabolites in C57BL/6 mice. Pharmacologist 22: 260

Wilson L. Bamburg JR, Mizel SB, Grisham LM, Creswell KM (1974) Interaction of drugs with microtubule proteins. Fed Proc 33: 158–166

Wilson L. Meza I (1968) Mechanism of action of colchicine. Colchicine binding properties of sea urchin sperm tail outer doublet tubulin. J Cell Biol 58: 709–719

Zweig G. Sharma J (1972) Handbook of chromatography V. II, Cleveland, p 162

Received August 4, 1980