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THE EFFECTS OF NATURALLY OCCURRING PHENOLICS ON THE ACTIVITY OF ORNITHINE DECARBOXYLASE IN C3H 10T½ CELLS: THE POSSIBLE INVOLVEMENT OF ACTIVATED SPECIES OF OXYGEN

by

Dolores Joyous Fatur

B.Sc. (Biochemistry Honors), Simon Fraser University, 1985

THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in the Department

of

Kinesiology

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July 1988

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ABSTRACT

The ability of phenolics to stimulate or inhibit ornithine decarboxylase activity in C3H 10T1/2 cells has potential as a screening procedure for tumor promoting/tumor inhibiting actions of phenolics. In a survey of several plant-derived phenolics, their addition to the culture media of C3H 10T1/2 fibroblast cells from mouse embryos had, in each case, a dramatic impact on the activity of ornithine decarboxylase (ODC). Three phenolics increased, four decreased and one increased and decreased the ornithine decarboxylase activity. The order of their ability to increase ODC levels was as follows; catechin (50-900μM) >>> gallic acid (gallate at neutral pH) (0.07-175μM) > catechol (30-500μM). The order of their ability to decrease ODC levels was as follows; eugenol (3-305μM) < caffeic acid (0.01-139μM) < quercetin (0.5-50μM). Ellagic acid (3-1660μM) exhibited both effects. These effects on ODC activity correlate well with previous reports of their potency in increasing or moderating the actions of carcinogens and tumor promoters. The ODC assay, therefore has potential as a screening procedure for the tumor promotion or protective actions of phenolics and perhaps other compounds.

To investigate whether active oxygen or transition metal ions play a role in the abovementioned ability of gallate to induce an increase in the activity of ODC, the cells were incubated for one hour with savengers of active oxygen or a metal chelating agent prior to the addition of gallic acid. Superoxide dismutase, catalase (both at 10 and 50U/ml) or D-mannitol (25 and 50mM) increased gallate's ability to induce ODC, while formic acid or benzoic acid (both at 50 and 75mM) completely prevented the stimulation of ODC activity by gallate. Desferrioxamine

(at 50 and 100μM) completely prevented the stimulatory effects of gallate.

When these same scavengers were compared with respect to their effects on the reactivity of gallate towards O_2 *in vitro*, the compounds which increased the rate of autoxidation also increased the effects of gallate on ODC activity. Conversely, compounds which slowed the autoxidation decreased these effects. Evidently intermediates in the oxidation of gallate rather than gallate itself are responsible for the induction of ODC activity. Since superoxide dismutase or catalase did not inhibit, these stimulatory intermediates are neither superoxide nor H_2O_2. That formic acid or benzoic acid moderated the gallate-induced increase in ODC activity suggests that the intermediates which mediate the gallate-induced increase in ODC activity, are hydroxyl radicals or related species.

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CHAPTER I

GENERAL INTRODUCTION

Polyamine function

Polyamines are a group of low molecular weight, long-chain, cationic aliphatic compounds with multiple amine and/or imino groups ubiquitous to all mammalian cells (Persson *et al* 1985). Levels of these organic cations: putrescine, spermidine and spermine fluctuate in parallel with DNA and RNA during chick embryo development (Russell and Snyder 1968) and are therefore thought to influence cellular growth and differentiation by affecting ribosomal and nucleic acid function. They are also thought to affect the metabolism and biological activity of proteins and phospholipids (Whaun and Brown 1985, Sadeh *et al* 1984 and Fidelus *et al* 1984).

Polyamine biosynthesis is one of the earliest events of cellular proliferation (Bachrach 1975). Increased production over that of non-proliferating cells has been observed in rapidly growing tissues such as embryonic tissue, regenerating liver, some tumors and normal tissues of tumor-bearing animals (Janne *et al* 1978, Russell and Snyder 1968, Heby and Russel 1973, Noguchi *et al* 1976). Consequently, much attention has been focused upon the biosynthesis of polyamines and the implications of their presence in normal and neoplastic tissue.

Of the four enzymes of the polyamine biosynthetic pathway, ornithine decarboxylase (E.C. 4.1.1.17) (ODC) is rate limiting in the formation of the polyamines and therefore holds a key position in the regulation of polyamine metabolism (Bachrach 1975).

Ornithine decarboxylase

The decarboxylation of L-ornithine in mammalian cells by ornithine decarboxylase is the only pathway for the production of putrescine (1,4-diaminobutane). This diamine is then used as a precursor for the formation of the two major polyamines spermine and spermidine (Heby *et al* 1975, Sekar *et al* 1983). Polyamine biosynthesis in mammalian cells is therefore solely dependent on the initial actions of ornithine decarboxylase. Since a relationship between ornithine decarboxylase activity and polyamine biosynthesis exists, (Haselbacher *et al* 1976, Chen *et al* 1976, Noguchi *et al* 1976) many investigators use ornithine decarboxylase activity instead of polyamine formation to indicate the proliferative state of a cell.

Because of the presumed association between ornithine decarboxylase and cellular proliferation and the observed increase in ornithine decarboxylase before the gross appearance of hyperproliferation (Connor and Lowe 1983), it is thought that ornithine decarboxylase levels fluctuate in a cell cycle-specific manner. Analysis of specific genes in the cell cycle has indicated that ornithine decarboxylase expression does in fact change throughout the cycle (Calabretta *et al* 1985). There is an increase of ornithine decarboxylase activity primarily during the G_1 phase (Heby *et al* 1976). If this increase does not occur the cell becomes quiescent and does not enter the S-phase (Thompson *et al* 1986).

Effects of growth-like factors on ornithine decarboxylase

Mitogens, tumor promoters and trophic hormones increase ornithine decarboxylase activity in a variety of tissue and cell lines. The regulation of ornithine decarboxylase is of special interest because this enzyme is induced by

a wide spectrum of growth stimulators, and shows remarkable variability in response to different stimuli. In cultured cells ornithine decarboxylase levels are enhanced by many peptide hormones, including growth hormone which elicited an increase in renal and hepatic ornithine decarboxylase activity (Nicholson *et al* 1977, Clark 1974). Addition of glucagon or insulin to isolated hepatocytes causes rapid transient increases in the enzyme activity, showing peak enzyme activities (100 fold and 25 fold control values, respectively) at 3.5 hours after administration. However, there is a rapid decline in activity to basal levels after this time (Lumeng 1979, Haselbacher and Humbel 1976).

Ornithine decarboxylase is one of the ten mammalian genes that undergo amplification under a selection pressure or appropriate cellular stress (Schimke 1984; Alhonen-Hongisto 1985). This increase in ornithine decarboxylase activity was thought to result from rising levels of cAMP. This possibility was investigated by introducing exogenous compounds which increased intracellular levels of this second messenger. The presence of dibutyryl cAMP or the cAMP phosphodiesterase inhibitor IBMX gave opposite effects. IBMX increased ornithine decarboxylase by 370 fold after 4 hours incubation with N115 neuroblastoma cells. Dibutyryl cAMP did not increase ornithine decarboxylase levels above control (Bachrach 1975). Although some of the stimuli (glucagon, epinephrine and isoproterenol) act via cAMP, this mechanism cannot account for the stimulatory effect of insulin, insulin-like stimulatory factor and epidermal growth factor which do not increase intracellular cAMP levels (Willey *et al* 1985). Cycloheximide in all the cases mentioned above, inhibited the induction of ornithine decarboxylase activity indicating that the stimulation is contingent on *de novo* mRNA synthesis (Lumeng 1979, Bachrach 1975). Thus increases in ornithine decarboxylase activity

may also be due to gene amplification, alterations in translation and enzyme stability (McConlogue 1986).

Effects of tumor promotors on cellular ornithine decarboxylase activity

Tumor promotors affect cells biochemically, changing their ability to alter the expression of many genes involved in growth and cellular differentiation (Gilmour *et al* 1987). The finding that tumor cells accumulate polyamines at certain stages of tumor growth (Heby and Russell 1973) lead to an investigation of the effect of tumor prmotors on ornithine decarboxylase activity. Many investigators have focused on the effects of the natural occurring complete tumor promoter, 12-O-tetradecanoylphorbol-13-acetate (TPA), found in croton oil, on ornithine decarboxylase (Lichti *et al* 1977).

The effect of TPA on the cellular ornithine decarboxylase activity is both dramatic and transient (Verma 1985b). Application of TPA to mouse epidermal cells both *in vitro* and *in vivo* produced a 200-fold transient increase in ornithine decarboxylase activity. The enzymatic activity peaked at 4.5 hours after application and returned to the normal basal levels by 24 hours. The levels of ornithine decarboxylase mRNA follows a similar pattern reaching a maximum at 3.5 hours and then decreases. The increase in ornithine decarboxylase activity is probably due, therefore, to alterations occurring at both the transcriptional and translational levels (Gilmour *et al* 1986, Gilmour *et al* 1987, Verma and Erickson 1986). Although the mechanism of the TPA induction of ornithine decarboxylase activity is not clear, evidence indicates that the epidermal induction of ornithine decarboxylase activity is an essential component of tumor promotion by TPA in cells.

Involvement of O_2^- in the induction of ornithine decarboxylase.

Mechanisms of the actions of tumor promoters such as TPA are not readily identified due to the complexity of the internal environment of the cell. The promoting actions of substances such as bleomycin and TPA seem to involve activated species of oxygen, since superoxide dismutase inhibits the promoter-induced neoplastic transformation of cultured cells (Borek and Troll 1983). This raises the question of whether superoxide also mediates the TPA decrease in ornithine decarboxylase activity.

In fact, the TPA induced ornithine decarboxylase activity in mammary tumor cells (Mm5mt/C1) can be suppressed by various antioxidants. While superoxide dismutase or catalase individually did not significantly decrease the effects of TPA on ornithine decarboxylase activity, the two scavengers in combination, or mannitol alone, inhibited the increase in ornithine decarboxylase activity by 50% (Friedman and Cerutti 1984). The cells *were not* preincubated with the antioxidant before adminstration of TPA, which may have led to a false negative. In order to provide a clearer picture of the role of active oxygen species, the cells should be either preincubated with the scavengers before TPA administration or the biomimetic analogs should be used (Kensler *et al* 1983). Cu(II) (3,5-diisopropylsalicylic acid)$_2$ (CuDIPS), a biomimetic analogue of SOD induced a 46% and 93% decrease in the number of papillomas per mouse when CuDIPS was administered 30 minutes before topical application of TPA (Kensler *et al* 1983).

A causal role of O_2^- in the induction of ornithine decarboxylase is disputed by Ghezzi *et al* (1986), who compared the macrophage cell line J774.16 with a mutant line which can not generate superoxide in response to TPA. They

reported a dissociation between stimulation of the oxidative burst and ornithine decarboxylase induction. Retinoic acid inhibits the TPA stimulation in ornithine decarboxylase activity, but it is unclear whether it acts as a free radical scavenger, or if it effects intracellular processes such as gene expression (Verma 1985, Verma and Erickson 1986).

Role of naturally occurring plant phenolics

Phenolic compounds are widespread in the environment. Both harmful and antineoplastic effects are attributable to plant phenolics (Clemo 1958; Stich *et al.,* 1981). Many studies have established the deleterious effects of phenolics in cigarette smoke and its link to lung cancer (Clemo, 1958). On the other hand plant phenolics, are potentially useful for the prevention of human disease as suggested by the antineoplastic effect of many plant preparations (Powell and Smith 1979). There is an inverse correlation between the consumption of vegetables and the incidence of cancers of the stomach (Graham *et al.,* 1972; Haenszel *et al.,* 1972; Haenszel *et al.,* 1976) colon (Modan *et al.,* 1975; Graham *el al.,* 1978; Haenszel *et al.,* 1980), and breast (Phillips 1975; Armstrong and Doll 1975).

Obviously a test to distinguish harmful from beneficial phenolics, will be useful. A practical predictor of the tumor promoting action of a phenolic may be its ability to stimulate ornithine decarboxylase activity. The following phenolics were selected for the study. 1) Quercetin which is one of the most ubiquitous flavonoids in the plant kingdom. It is found in fruits and vegetables and is particularly high in clover blossoms and ragweed potten. 2) Ellagic acid, which also occurs in a wide variety of green plants. Major dietary sources of

this aromatic phenolic include coffee, grapes and nuts. 3) Caffeic acid [3,4-dihydroxycinnamic acid] which is found in plants in a conjugated form is readily hydrolyzed to yield free caffeic acid. It is readily isolated from green coffee, roasted coffee, vegetables such as red and white cabbage and celery. 4) Eugenol which is an active component in dental analgesic medicines 5) catechin [trans-2-(3,4-dihydroxyphenyl)-3,4-di-hydro,2H-1-benzopyran-3,5,6-triol] which is found primarily in woody plants and in cigarette smoke. 6) Gallic acid which is found free, and complexed in the form of tannins, in a wide variety of plant species. It is particularily abundant in Japanese green tea leaves and can be obtained in its complexed form from the tannins of nutgalls (excrescences upon a variety of plant species). Its presence in grapes means that it is also found in high concentrations in red and white wine. Lastly, 7) catechol which is found in coffee and is reportedly the major constituent of cigarette smoke.

Perspective

The correlation between suppression of TPA induced ornithine decarboxylase activity and prevention of TPA enhanced papilloma formation suggests that ornithine decarboxylase induction may be an important mechanism in promotion. Hence the ability of a compound to increase (or suppress an increase) in ornithine decarboxylase activity may indicate that it is a tumor promoter or inhibitor. Because carcinogenesis is a multi-stage process involving many environmental factors, it would be of great interest to study a variety of naturally-occurring compounds for their effects on ornithine decarboxylase. In the first part of this study, we assess the effects of various naturally occurring phenolics on the activity of ornithine decarboxylase in C3H 10T1/2 cells. These data were compared with available mutagenic and clastogenic data, with the hope

of establishing the system as an assay procedure for putative tumor promoters and inhibitors. It is also hoped that this study will provide a model for predicting the inhibitory and stimulatory effects of the phenolics on ornithine decarboxylase, based on their structures.

Scavengers of active oxygen species prevent the enhancement of papilloma formation by TPA. In the second part of the study therefore we investigate the role of oxygen free radicals and metals as mediators in enhancing the phenolic-induced stimulation of the ornithine decarboxylase activity. Gallic acid was chosen for this study since free radicals are produced during its autoxidation and data indicating the effects of various scavengers of active oxygen species on the rate of oxidation was available.

CHAPTER II

GENERAL MATERIALS AND METHODS

Reagents

DL-[1-^{14}C] Ornithine (61 mCi/mmol) was obtained from New England Nuclear. 12-O-tetradecanoylphorbol-13-acetate (TPA), pyridoxal 5'-phosphate, L-ornithine·HCL, disodium ethylenediaminetetracetate (EDTA), citric acid monohydrate, caffeic acid [3,4-dihydroxycinnamic acid], quercetin, (+)-catechin [trans-2-(3,4-dihydroxy-phenyl)-3,4-di-hydro, 2H-1-benzopyran-3,5,7-triol], ellagic acid [4,4',5,5',6,6'-hexa-hydrodiphenic acid 2,6,2'6' dilactone], bovine serum albumin, superoxide dismutase EC 1.15.1.1, from bovine blood, (3100U/mg protein) lot #93F-9305, catalase EC 1.11.1.6, from bovine liver, (11000U/mg protein) lot #82F-0838, formic acid (sodium salt), benzoic acid (sodium salt), dithiothreitol, and D-mannitol were all from Sigma Chemical Co., St. Louis, Mo. Tris(hydroxymethyl)methylamine, and 2-methoxyethanol, were from BDH Chemicals, Vancouver, B.C. Ethanolamine was from Fisher Scientific, Fair Lawn, N.J. Gallic acid [3,4,5-trihydroxybenzoic acid] and catechol were from Aldrich Chemical Co., Milwaukee, Wis. Bradford protein assay ~~dye~~ was from Bio-Rad Laboratories, Richmond, Calif. Desferrioxamine (desferal mesylate) was a gift from CIBA Pharmaceutical Co., Summit, N.J.

Cell Line

The contact sensitive mouse embryo cell line, C3H 10T1/2, passage 8, was obtained from American Type Culture Collection, Rockville, Maryland. Cells for experimental purposes were grown in Nunclon plastic dishes of 100mm in diameter in Dulbecco's modified Eagle's medium "growth medium" (Gibco Laboratories). This was supplemented with 10% (v/v) fetal bovine serum (FCS),

(Flow Laboratories, McLean Virginia), and 20mM HEPES [N-2Hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Sigma Chemical Co.). Stock cultures were maintained in 80cm² Nunclon flasks. Both the 80cm² flasks and the 100mm dishes were seeded at the same density (approximately 120 x 10^4 cells/plate) and allowed to reach confluency over five days, with no change of growth medium. The cultures were incubated at 37°C at 5% CO_2 in a humidified atmosphere.

Trypsinization procedures for this particular cell line were optimized, and all subsequent trypsinizations were then carried out systematically; cell medium was poured off and the cells were washed once with 0.05% trypsin (Gibco) dissolved in phosphate buffered saline (0.8% NaCl; 0.02% KCl; 0.115% Na_2HPO_4; 0.02% KH_2PO_4; 0.02% EDTA). The cells were then incubated with 0.05% trypsin in phosphate buffered saline for 30 seconds and the trypsin poured off; residual trypsin was allowed to stay on the cells for an additional 45 seconds. The cells were then resuspended in Dulbecco's modified Eagle's medium and the cell density was determined using a Bright-line hemacytometer. This entire procedure was performed under sterile conditions in a laminar flow hood.

Cell Treatment

All treatments were applied to a confluent monolayer of cells in a 100mm dish, with no media changes during the 5 days between subculture and treatment. Stock solutions of the appropriate chemicals were made up in growth medium. The appropriate volume was then added directly to the 10ml of a 5 day old conditioned medium on the plates. After treatment the cells were returned to the 37°C incubator.

After the predetermined exposure period, the medium was removed by aspiration and the monolayer was washed twice with cold phosphate buffered saline. The cells were removed from the plates (using a rubber-policeman) resuspended in phosphate buffered saline, and transferred to 5ml Falcon tubes. The cells were centrifuged at 50 revolutions per minute (RPM) for 5 minutes, the phosphate buffered saline was then removed and the pellet of cells was stored at -70°C until ornithine decarboxylase enzyme activity assay could be performed.

Ornithine Decarboxylase Activity Assay

The cell pellet was thawed at room temperature and resuspended in assay buffer. The assay buffer contained 25mM Tris-HCL (pH 7.5), 25μM pyridoxal 5'-phosphate, 2.5mM dithiothreitol, and 50μM EDTA. The cells were then disrupted by three successive cycles of freezing and thawing, and the cellular debris was removed from the cellular extract by a 10 minute centrifugation at 12 x 10^3 RPM. The clear supernatant was removed and incubated for 10 minutes at 37°C.

The ornithine decarboxylase activity of the supernatant was determined by measuring the release of $^{14}CO_2$ from the enzymes' substrate, DL-[1-^{14}C]ornithine hydrochloride (Feo *et al* 1985). The reaction was initiated by the introduction of 250μl of cellular extract into the eppendorf tubes which already contained L-ornithine (0.77μM).

Once initiated, the entire assembly was incubated in a 37°C water bath for 1 hour. The reaction was then terminated and CO_2 was liberated from the center well by the injection of 500μl of 2M citric acid through the rubber stopper. To ensure complete absorption of CO_2 by the ethanolamine and methoxyethanol

(100μl; 2:1 v/v) (which is located at the bottom of the scintillation vial) i.e., the outer compartment of the assembly (refer to figure 2.1). The assembly was left at room temperature for an additional 18 hours. Finally, the eppendorf tube was removed and the outside wall of the eppendorf tube was rinsed with 1ml ethanol (to wash off any ethanolamine:methoxyethanol which might have come into contact with the outside of the eppendorf tubes). The center wells were then capped and discarded. Five ml of toluene-based scintillation fluid was added to the scintillation vial and mixed. The radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer which counted each tube for 10 minutes.

A given cell treatment on any one day was done in triplicate (3 separate 100mm dishes were exposed to the same conditions). Two ornithine decarboxylase determinations were performed on the cell extract obtained from each dish. Thus, for each cell treatment, on any given day, a total of six ornithine decarboxylase activity measurements were taken, figure 2.2.

The protein content of the cellular extract was determined by the Bio-Rad procedure using bovine serum albumin as the protein standard (Bradford 1976). The Bio-Rad assay is based on differential color changes of the dye Coomassie Brilliant Blue to varying concentration of proteins. The binding of protein to the dye shifts the absorbance maximum of the dye from 465nm to 595nm. Protein content of an unknown was determined by spectrophotometric measurements at 595nm and estimated by comparison to the results obtained with known concentrations of bovine serum albumin (Spector 1978) ranging from 0.02-0.140 mg/ml.

Colony Forming Efficiency (CFE)

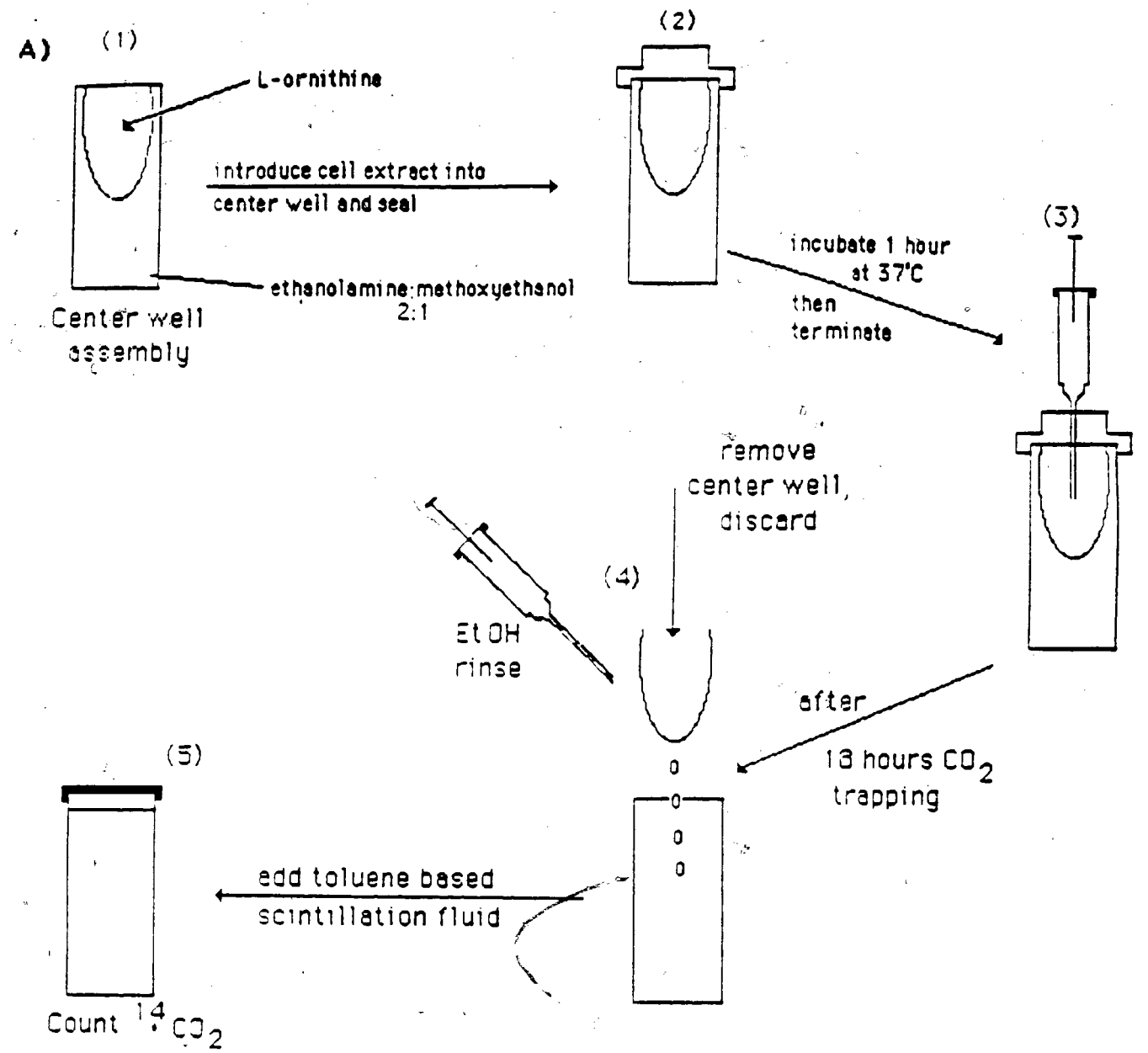
Starting at approximately 4:30 pm the day before the addition of phenolic, a stock flask of C3H-10T1/2 cells was trypsinized and seeded onto dishes of 60mm diameter at a density of 150 cells per plate. At approximately 9 am the following day the cells were exposed to the appropriate chemical for 5 hours. The medium was then removed, the plate was washed once with growth medium containing no fetal calf serum, followed by the addition of fresh growth medium containing 10% fetal calf serum. The cells were allowed to grow at 37°C for 5 days. The medium was then removed and the plates were rinsed with distilled water. The cells were then fixed and stained with 0.1% methylene blue in 50% methanol for at least 10 min. The cells were then rinsed with distilled water and air dried. The colonies were counted under a dissecting microscope.

Legends For Figures

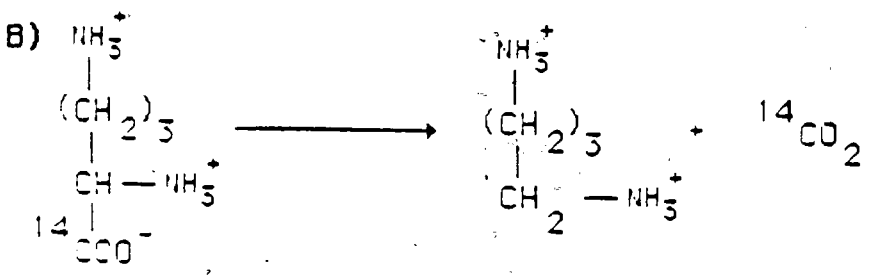
Figure 2.1 Schematic representation of the assay procedure used for ornithine decarboxylase activity determination

Figure 2.2 Scheme of cell treatment for the ornithine decarboxylase assay

FIGURE 2.1



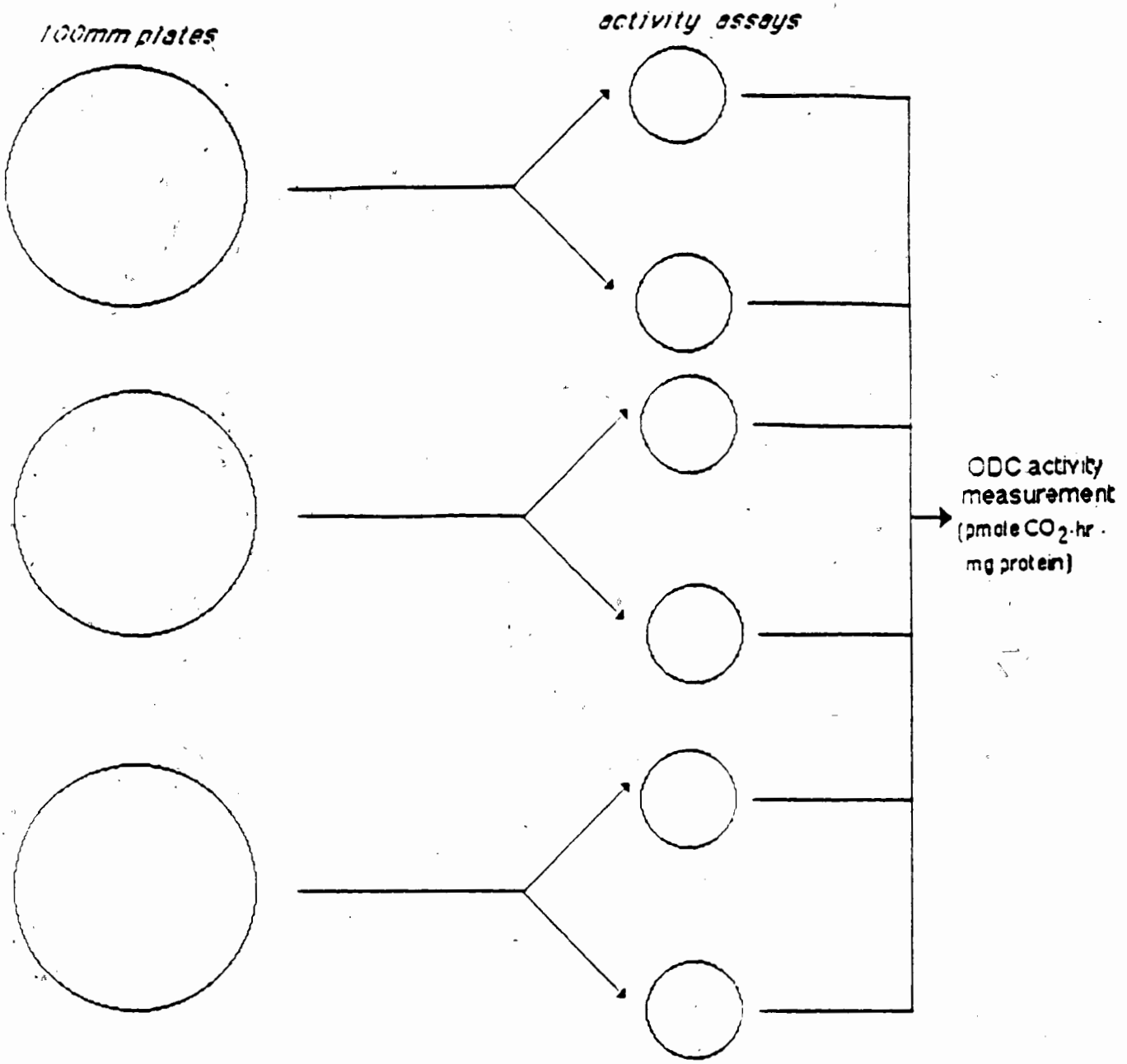
B)



Ornithine

Putrescine

FIGURE 2.2



In summary, 2 activity determinations were performed on cell extract obtained from each plate, 3 plates are treated identically, therefore 6 activity measurements represent one treatment.

These are duplicated on a different day so that any one condition reported is indicative of a comparison of at least 12 activity measurements.

CHAPTER III

OPTIMIZING EXPERIMENTAL CONDITIONS

Preliminary experiments were undertaken to optimize the ornithine decarboxylase assay. The objectives were to establish: (1) the best method for releasing cellular extract from the cells, (2) a saturating concentration of substrate in the reaction mixture, and (3) an optimal time for trapping all the $^{14}CO_2$ released in the reaction catalysed by ornithine decarboxylase. We also established cell-handling procedures which provided low basal activities of ornithine decarboxylase.

Sonication and freeze thawing were compared as methods for disrupting cellular membranes. Sonication was carried out as follows; the cells were harvested and resuspended in assay buffer in a 5ml Falcon tube. The Falcon tube was placed in an iced water bath and a sonicating probe was inserted into the tube. The cells in the tube were subjected to 3 cycles of a 15 s pulse of sonication at a setting of 30 using a Bronwill Biosonik III sonicator, with a 30 s pause between each pulse. The "freeze/thaw" technique was as follows; cells were resuspended in the assay buffer and subjected to three cycles of freezing and thawing. In each cycle the cells were allowed to freeze in a dry ice-95% ethanol bath for 1 minute. Thereafter they were removed and submerged in a 37°C water bath for 4 minutes to ensure thawing.

The activities of ornithine decarboxylase recovered by each technique were compared. Figure 3.1 shows that cell extracts obtained by the "freeze/thaw" method had a 3-fold greater ornithine decarboxylase activity than did those subjected to the sonication method. The reproducibility of the freeze thawing

technique was evident by the high degree of consistency between the trials (each trial represented by one bar). We therefore chose to disrupt the cells in all subsequent experiments by the freeze thawing method described above.

To determine that the reaction mixture contained a saturating concentration of substrate, extracts from cells exposed to TPA (100ng/ml final) for 5 hours were incubated with a range of substrate concentrations (0.33-4.6μM). Figure 3.2 showed that the activity reached a maximum value when the ornithine concentration was 0.76μM or greater. We therefore chose to include 0.77μM ornithine in the assay mixture.

We determined the time required for the ethanolamine:methoxyethanol mixture to absorb all the CO_2 released from the enzyme reaction. Ten assemblies were prepared containing the same reaction mixture, namely 25mM Tris.HCL, 25μM pyridoxal 5'-phosphate, 2.5mM dithiothreitol, and 50μM EDTA. After incubation at 37°C for one hour the reaction was stopped by the injection of 500μl of 2M citric acid. The assemblies were opened one by one at hourly intervals and the amount of CO_2 absorbed determined. We found that at least 15 hours is required for a complete absorption of the CO_2 released in an enzyme reaction. To ensure that there was enough air movement in the assembly to move the CO_2 from the center well into the outer chamber to be trapped, an experiment was performed where convection currents were set up inside the assembly (by heating one side of the apparatus and keeping the other cool). It was found that creating these convection currents did not increase the amount of $^{14}CO_2$ trapped over that which was trapped after 15 hours of room temperature incubation. We therefore routinely allowed the assembly to stand at room temperature for 18 hours or longer before we determined the $^{14}CO_2$ trapped.

To obtain a low basal level of ornithine decarboxylase, C3H 10T1/2 cells were subcultured on day zero at a density of 1.2 $x10^6$ cells/100mm plate. Cells were allowed to grow under the standard conditions (refer to materials and methods) for 1 to 6 days following subculture with no medium change. The cells were then harvested and stored at -70°C. The ornithine decarboxylase activity in the cells harvested on different days were compared. Figure 3.3 indicates that not only did the basal levels of ornithine decarboxylase decrease as the cells approached confluency (compare bars from day one with those of days 5 or 6), but the consistency of ornithine decarboxylase activity per plate increased (each bar represents the results of one plate). In view of this we chose to chemically treat the cells five days after subculture with no medium change between subculture and treatment.

It has been shown that the cellular response can change with the "age" (i.e., the passage number) of a cell line (Rozhin, et al, 1984). It was our concern therefore to ensure that the cellular responses remained statistically similar between the different passage numbers of C3H 10T1/2 cells. Cellular responses to a 5 hour incubation of TPA (100ng/ml final) were monitored in cells ranging in age from passage 12 to 22. It is evident from figure 3.4(i) that the absolute value of ornithine decarboxylase activity of control and TPA treated cells *did* vary from passage to passage. However the percent increase in ornithine decarboxylase activity induced by the treatment did not seem to fluctuate greatly as the cells aged (figure 3.4(ii)).

To ensure a reproducible and maximal response of ornithine decarboxlase to gallate, preliminary trials to optimize the preparation and cellular incubation time were carried out. Solutions of gallate were prepared both anaerobically and

aerobically in double distilled water, phosphate buffered saline or serum free Dulbecco's modified Eagle's medium. There were no significant differences in the gallate-induced ornithine decarboxylase activity among the different solutions used to dissolve the phenolic, nor was there a significant difference in the results from anaerobically versus aerobically prepared gallate. The gallate stock solution was therefore prepared under aerobic conditions in double distilled water.

The cellular incubation time was optimized by exposing a number of plates of C3H 10T1/2 cells to gallate (prepared as described above) for different times. After the exposure time the gallate containing medium was removed and replaced with fresh serum containing Dulbecco's modified Eagle's medium. All of the treated cells (regardless of the exposure period) were harvested 5 hours after the exposure to gallate was initiated. Refeeding the starved cells with serum rich medium, after gallate was removed, greatly increased the levels of ornithine decarboxylase activity (c.f. the ornithine decarboxylase activity of nontreated cells with no medium change was 101 pmole CO_2 released per hour per mg protein, whereas nontreated cells with a medium change had an ornithine decarboxylase activity ranging from 1170-2966). This large ability of serum to increase ornithine decarboxylase partially masked the effects of gallate. We therefore concluded that the best response was obtained when gallate was incubated with the cells for the entire five hour period with no medium change (figure 3.5).

Legends For Figures

Figure 3.1 Ornithine Decarboxylase activity as a function of the method used to disrupt cells

C3H 10T1/2 cells were subjected to freezing followed by thawing or sonication. The freeze-thaw method involved subjecting the cells to three successive cycles of freezing (dry ice-95% ethanol bath for 1 min), and thawing (submerged in a 37° water bath for 4 min). The sonication technique involved 3 cycles of a 15 s pulse. The assay condition were the same as those described earlier in the section headed "Assay for Ornithine Decarboxylase Activity". Ornithine decarboxylase activity was measured by the release of $^{14}CO_2$ from ^{14}C-L-ornithine, and the protein content was determined by the Bio-Rad protein assay. The activity is expressed as pmole of CO_2 released per hour per mg protein. Each bar represents one sample.

Figure 3.2 Saturating substrate concentration

Cellular extracts of C3H 10T1/2 cells treated with TPA (100ng/ml final) for 5 hours were incubated with increasing substrate concentration. Conditions were as indicated for figure 3.1. The enzyme activity is reported as pmole of CO_2 released per hour incubation per mg protein.

Figure 3.3 Basal levels of ornithine decarboxylase as a function of number of days from subculture

C3H 10T1/2 cells were seeded onto 100mm plates on day zero. On day one (one day after subculture) six plates were scraped and the cells were isolated and stored at -70°C. This procedure was carried out daily up until and including day 6. Conditions were as indicated for figure 3.1. The ODC activity was determined and reported as pmole CO_2 released per hour per mg protein. Each

point on the graph represents one plate of cells.

Figure 3.4 Effect of cell age on ODC response absolute values and values relative to control

3.4(i) crosses represent the ODC activity (pmole CO_2 released/hr/mg protein) of control cells i.e., no treatment

diamonds represent the ODC activity (pmole CO_2 released/hr/mg protein) of cells after a 5 hour treatment of TPA (100ng/ml final). The plates containing control and TPA treated cells were isolated and frozen on the same day. The assay conditions for the determination of ornithine decarboxylase were as described for figure 3.1. Each mark represents one plate of cells.

3.4(ii) The condition were the same as in 3.4(i). The % change from control represents the percentage difference between treated and nontreated cells ((treated - nontreated/ nontreated) x 100). Each bar represents one plate of cells.

Figure 3.5 The effect of duration of exposure to gallate on ornithine decarboxylase activity

The conditions were as indicated in figure 3.1. The duration of exposure to gallate refers to the time (in minutes) that the cells were exposed to gallate. After exposure the gallate containing medium was removed and the cells were "refed" with fresh serum containing medium. The cells were then incubated with fresh medium until the exposure time plus incubation with fresh medium time equalled 300 minutes. The cells were isolated and ornithine decarboxylase activity determined. The % change from control is as indicated in figure 3.4(ii). Each bar represents the average of two plates.

FIGURE 3.1

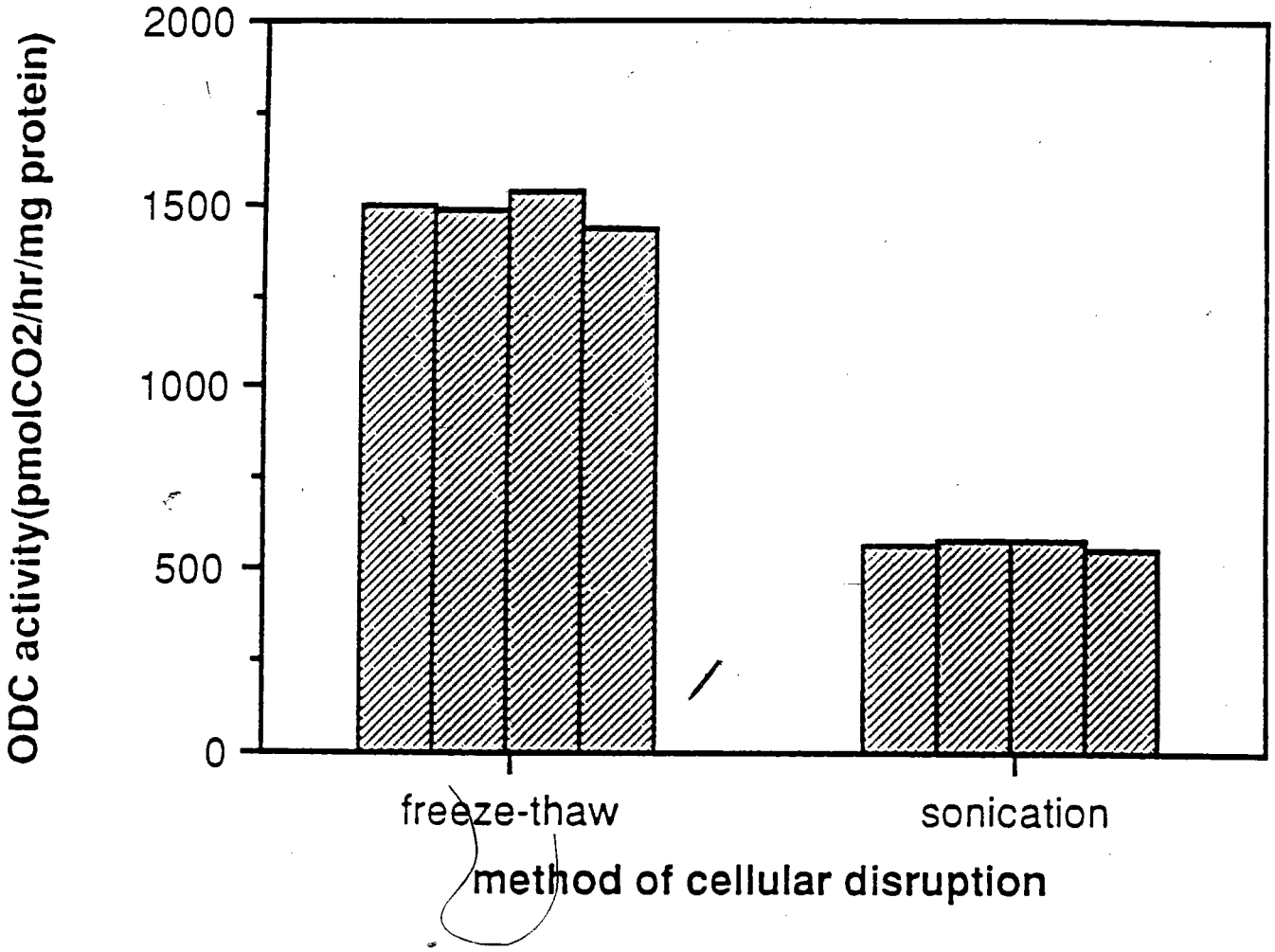

ODC activity(pmolCO2/hr/mg protein)
2000
1500
1000
500
0
freeze-thaw
sonication
method of cellular disruption

FIGURE 3.2

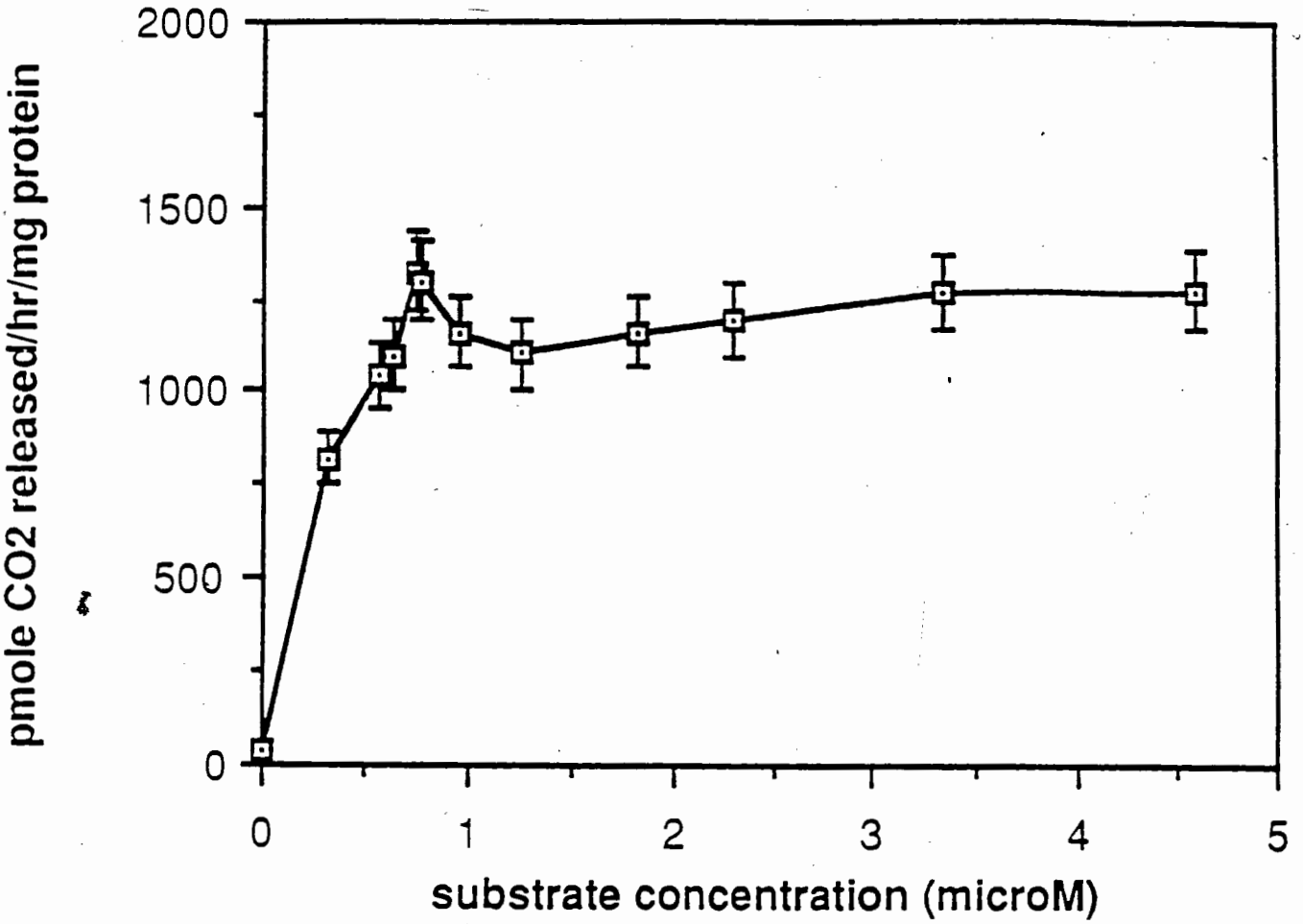

2000
1500
1000
500
0
pmole CO2 released/hr/mg protein
0
1
2
3
4
5
substrate concentration (microM)

FIGURE 3.3

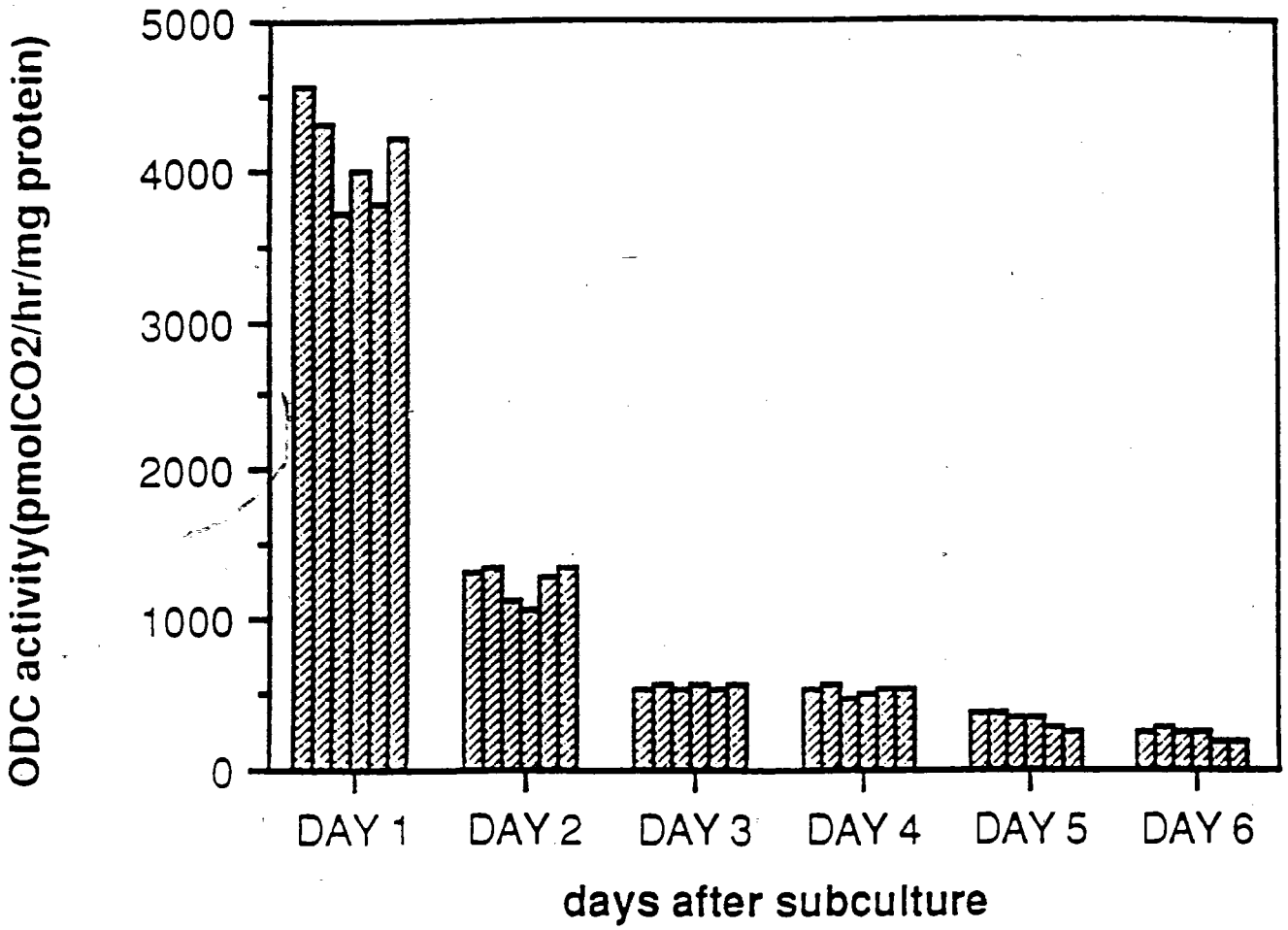
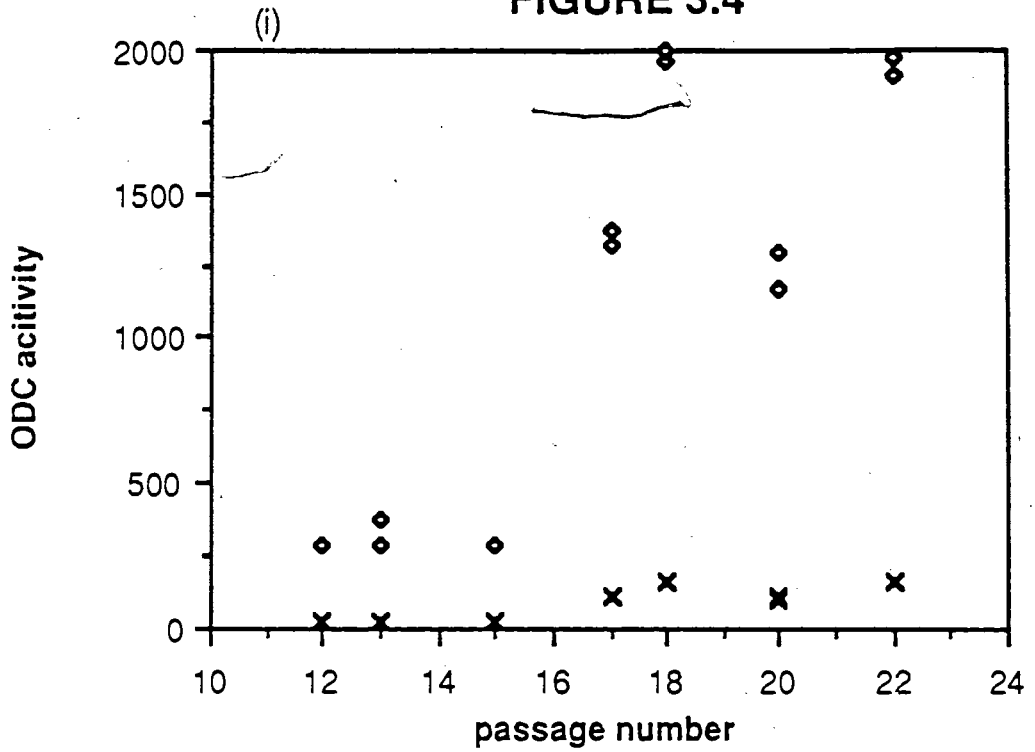

ODC activity(pmolCO2/hr/mg protein)
5000
4000
3000
2000
1000
0
DAY 1
DAY 2
DAY 3
DAY 4
DAY 5
DAY 6
days after subculture

FIGURE 3.4


(i)
2000
1500
1000
500
0
ODC acitivity
10
12
14
16
18
20
22
24
passage number

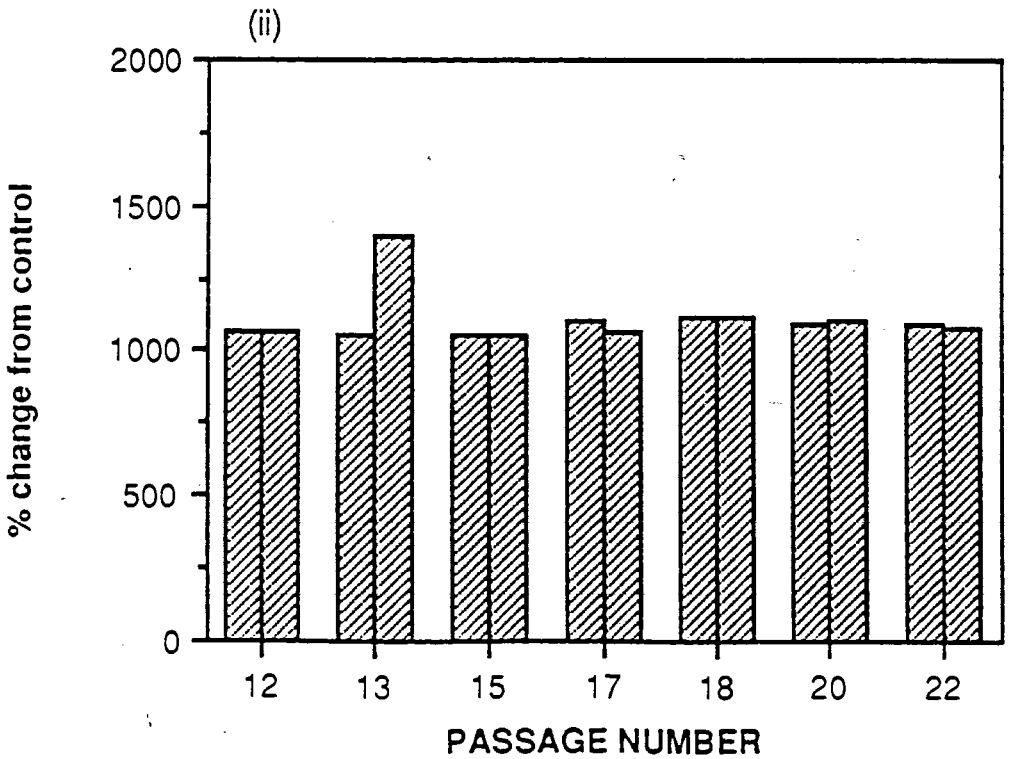
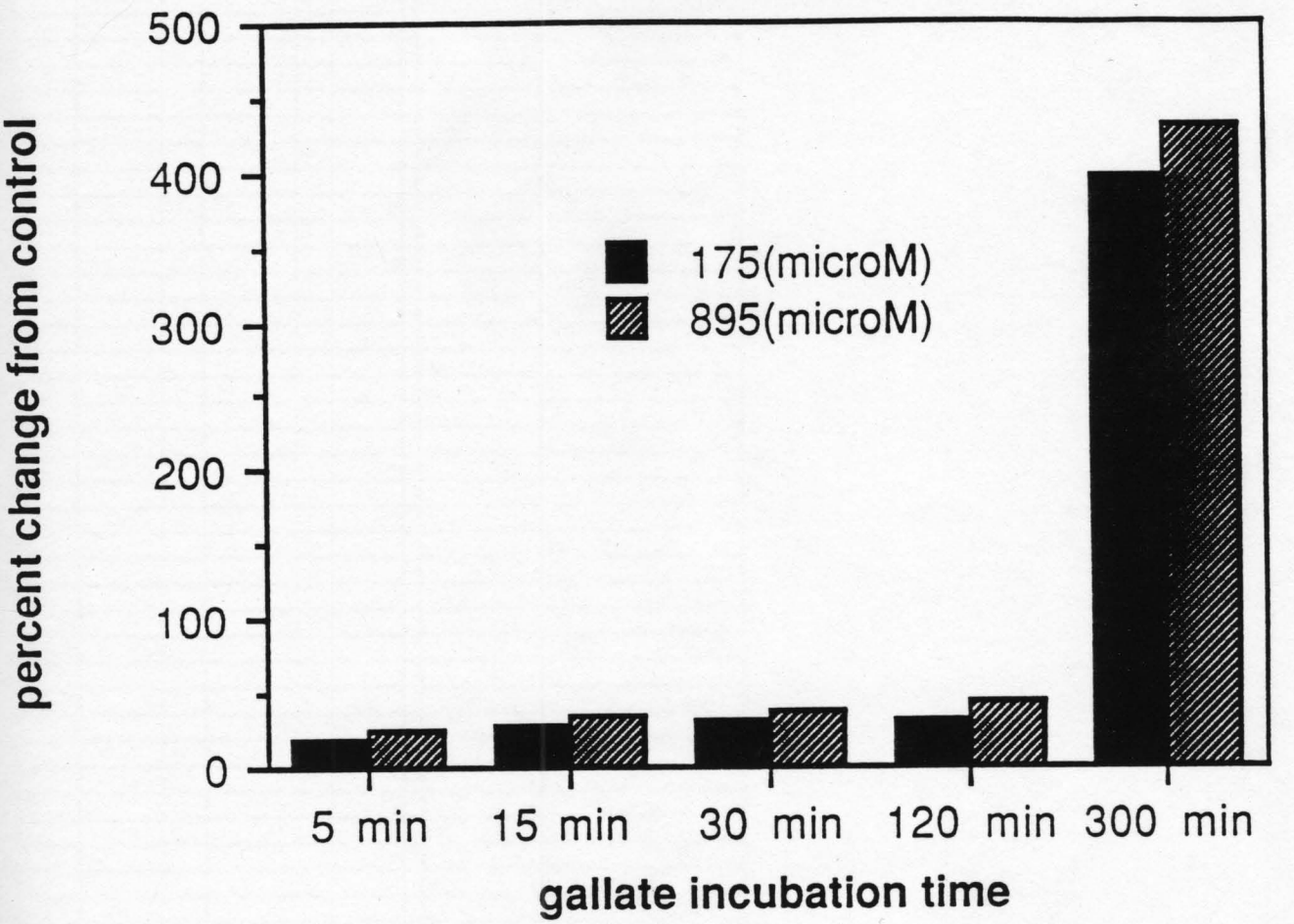

(ii)
2000
1500
1000
500
0
% change from control
12
13
15
17
18
20
22
PASSAGE NUMBER

FIGURE 3.5


500
400
300
200
100
0
percent change from control
175(microM)
895(microM)
5 min
15 min
30 min
120 min
300 min
gallate incubation time

CHAPTER IV

THE EFFECTS OF PLANT PHENOLICS ON THE ACTIVITY OF ORNITHINE DECARBOXYLASE IN C3H 10T1/2 CELLS

ABSTRACT

Addition of plant-derived phenolics to the culture media of mouse embryo fibroblast cells (C3H 10t1/2) had a dramatic impact on the activity of ornithine decarboxylase (ODC). Catechin (50-900μM) increased ODC activity to a maximum of 2200% above control at 90μM. The ODC response to the addition of gallic acid (0.07-175μM) reached a maximum increase of 400% at 160μM. Similarly, catechol (30-500μM) reached a maximum increase of 350% at 300μM or above. Other phenolics (eugenol, 3-305μM; caffeic acid, 0.01-139μM; and quercetin, 0.5-50μM) at all concentrations tested decreased the enzyme activity by as much as 27.8%, 31.5%, and 48.1%, respectively. Ellagic acid was the only phenolic tested which had both inhibitory and stimulatory effects on ODC activity. At concentrations of 3-331μM it decreased the activity of ODC, inhibiting maximally (43% inhibition) at 331μM. Higher concentrations had no inhibitory effect but instead stimulated, up to 12% above control at 1660μM ellagic acid. The order of effectiveness in stimulating or inhibiting ODC activity was thus dihydroxylated phenols (ortho more than meta), < trihydroxylated phenols, < tetra and penta hydroxylated phenols. This trend coincides with reported effectiveness in carcinogenesis as well as with the ability of similar phenolics to induce chromosomal aberrations in cultured cells. Introduction of an alkyl group to the ring, or replacement of a hydroxyl group by a methyl ester decreases the ability of a compound to induce ornithine decarboxylase.

INTRODUCTION

Both harmful and antineoplastic effects are attributable to plant phenolics (1,2). Many studies have showed the deleterious effects of phenolics in cigarette smoke and their link to lung cancer (1). Antineoplastic effects of plant preparations, which may in part be due to phenolics, are potentially useful for the prevention of human disease (3). For example, there is an inverse correlation between vegetable consumption and the incidence of cancers of the stomach (4,5,6), colon (7,8,9), and breast (10,11), which may in part be due to vegetable phenolics.

Because phenolic compounds are widespread in the environment, their apparent harmful and protective effects have aroused much interest. Many investigators have examined the clastogenicity, mutagenicity, tumor promoting, and antioxidant nature of phenolics (2,12,13,14,15,16). Various phenolic compounds induce chromosomal breaks in the chinese hamster ovary (CHO) cells, while others decrease the number of chromosome breaks per cell (2). On the basis of the *Salmonella typhimurium* test, some phenolics protect the bacteria against the mutagenic actions of 7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetra-hydrobenzo[a]pyrene (12), N-methyl-N'-nitro-N-nitroguanidine (MNNG) (13,14), methyl-nitrosourea (MNU) and 2-aminoanthracene (2-AA) (15,16).

Although many flavonoids are mutagenic (17), they are not carcinogenic (18,19,20). However, administration of 10μg/ml of quercetin to Chinese Hamster Ovary (CHO) cells did not increase chromatid breaks or exchanges (15). In fact, prior application of quercetin (10-30μmol/mouse) to mouse epidermis decreases the number of 12-O-tetradecanoylphorbol-13-acetate (TPA) induced papillomas per

CD-1 mouse by 71% (18). Moreover, in teleocidin promoted cells, quercetin (30μmol/mouse) decreases the number of papilloma formation per mouse by 83% while also decreasing the size of the tumors (21).

Ellagic acid has a very profound inhibitory effect on the mutagenic and cytotoxic actions of the carcinogenic metabolite of benzo[a]pyrene, and is the putative prototype of a new class of cancer-preventing agents (22). The presence of ellagic acid also decreased, in a dose-dependent manner, the amount of mutagenicity (induced by benzo[a]pyrene 7,8-diol-9,10-epoxide-2) in TA100 cells by as much as 90% at an ellagic acid concentration of 6μM (12). The antimutagenic actions of this phenolic supposedly result from a direct interaction with benzo[a]pyrene 7,8-diol-9,10-epoxide-2 (23).

In contrast, catechin (30μmol) did not inhibit either the TPA-induced ornithine decarboxylase activity in mouse epidermis, or the formation of TPA-induced skin papillomas (24), (although in the former studies positive effects of catechin may have been masked by the even larger effect of TPA). However, catechin in millimolar concentration, does inhibit the mutagenicity of methyl-nitrosourea in the TA1525 strain (13). In mammals (Syrian golden hamsters) oral administration of catechol (at 1.5% of diet) caused epithelial damage and consequent regenerative hyperplasia in the pyloric region, but its administration did not lead to severe hyperplasia or tumorous lesions in the forestomach (25). In mouse skin however, catechol (2mg per applications) enhanced the carcinogenicity of benzo[a]pyrene, more than doubling the number of mice with papillomas over control (26).

There is some structural basis for the net proxidant or antioxidant effect of the phenolics. Quercetin and three other structurally similar flavonoids: morin,

fisetin and kaempferol all moderated the TPA-induced increase in ornithine decarboxylase activity in the order: morin (95% inhibition)>fisetin=kaempferol (86% inhibition)>quercetin (76% inhibition) (18,24). Studies with compounds structurally similar to eugenol and caffeic acid (p-coumaric and ferulic acid) indicate that compounds with two hydroxyl groups on the ring are more potent in causing chromosomal aberrations and mutagenesis (13) than those with one hydroxyl (compare effects of p-coumaric (6mg/ml) and caffeic acid (0.2mg/ml)). Dihydroxylated phenolics are also more potent in causing chromosomal aberrations than those with one hydroxyl group plus a methyl ester (compare caffeic (0.2mg/ml) to ferulic (25mg/ml) acid and eugenol (0.4mg/ml)) (2). Methyl esters decrease the protective nature of a compound towards the mutagenicity of benzo[a]pyrene (12). An ortho diphenol orientation is more potent for stimulation (or lack of inhibition) than the meta orientation (compare catechol, resorcinol and hydroquinone) while para-hydroxyl substitution is the least potent in inducing hyperplasia in hamster forestomach (25) and chromosomal aberrations (15).

Despite these stuctural correlations, however, it is not yet possible to distinguish harmful from protective phenolics on the basis of their structure, although the usefulness of such a distinction is apparent. In this regard the ability of a phenolic to increase the activity of ornithine decarboxylase enzyme may be a useful predictor of its tumor promoting action. An increase in activity of this enzyme is one of the earliest events of cellular proliferation in cell culture and in animal models (27). Growth hormone, glucagon, insulin as well as tumor promoters, all increase the activity of ornithine decarboxylase (28,29,30,31).

Because of the stimulatory effects of tumor promoters on ornithine decarboxylase activity, a number of investigators suggest that the induction of

this enzyme has a causal role in tumor promotion (32,33). Many antioxidants (33), phenolics (19,24,25,34) and flavonoids (18,24) have been administered concurrently with tumor promoters and the effects on ornithine decarboxylase activity reported. The results to date suggest that the enhancement or suppression of ornithine decarboxylase activity may be useful as a short-term screening process for potential promoters and inhibitors of tumorigenesis (32).

In the current study therefore, we report the effects of various naturally occurring phenolics on the activity of ornithine decarboxylase in C3H 10T1/2 cells. Our objective was to determine whether the assay would prove to be a reliable method for the prediction of the tumor promoting and inhibitory actions of the phenolics. It was also hoped that the results would suggest a structural basis for the inhibitory and stimulatory effects of the phenolics on ornithine decarboxylase activity.

MATERIALS AND METHODS

Reagents

DL-[1-^{14}C] Ornithine (61 mCi/mmol) was obtained from New England Nuclear. 12-O-tetradecanoylphorbol-13-acetate (TPA) lot #34F-0682, pyridoxal 5'-phosphate, L-ornithine·HCL, disodium ethylenediaminetetracetate (EDTA), citric acid monohydrate, caffeic acid [3,4-dihydroxycinnamic acid], quercetin, (+)-catechin [trans-2-(3,4-dihydroxyphenyl)3,4-di-hydro, 2H-1-benzopyran-3,5,7-triol], ellagic acid [4,4',5,5',6,6'-hexahydrodiphenic acid 2,6,2'6' dilactone], and bovine serum albumin (lot #25F-0057), were from Sigma Chemical Co., St. Louis, Mo. Tris(hydroxymethyl)methylamine, and 2-methoxyethanol, were from BDH Chemicals, Vancouver, B.C. Ethanolamine was from Fisher Scientific, Fair Lawn, N.J. Gallic acid [3,4,5-trihydroxybenzoic acid] and catechol were from Aldrich Chemical Co., Milwaukee, Wis. Bradford protein assay dye lot #31751 was from Bio-Rad Laboratories, Richmond, Calif.

Cell Line

The contact sensitive mouse embryo cell line, C3H 10T1/2, passage 8, was obtained from American Type Culture Collection, Rockville, Maryland. Cells for experimental treatment, were grown in Nunclon 100mm plastic dishes in Dulbecco's modified Eagle's medium (DME lot #6OK1862) obtained from Gibco Laboratories Grand Isle., New York. This was supplemented with 10% (v/v) fetal bovine serum (FCS, select silver lot #29161018), Flow Laboratories, McLean Virginia, and Hepes [N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid] (lot #46F-5625S), cell culture tested, (20mM) final concentration from Sigma Chemical Co.

Stock cultures were maintained in 80cm² Nunclon flasks. Both the 80cm² flasks and the 100mm dishes were seeded at the same density (approximately 120 x 10^4 cells/plate) and allowed to reach confluency over five days, with no change of medium. Although the stock cultures and the 100mm dishes were stored in different incubators, the environment in both incubators was closely regulated at 37°C, 95% air/5% CO_2.

For this particular cell line, duration of trypsinization was optimized, and all subsequent trypsinizations were then carried out systematically; cell medium was poured off and the cells were washed once with 0.05% trypsin (porcine parvovirus tested, lot #72P4615 obtained from Gibco) dissolved in phosphate/saline (0.8% NaCl; 0.02% KCl; 0.115% Na_2HPO_4; 0.02% KH_2PO_4; 0.02% EDTA). The cells were then incubated with 0.05% trypsin in phosphate/saline for 30 seconds and the trypsin was poured off; residual trypsin was allowed to stay on the cells for an additional 45 seconds. The cells were then resuspended in supplemented Dulbecco's modified Eagle's medium and the cell density was determined using a Bright-line hemacytometer. This entire procedure was performed under sterile conditions in a laminar flow hood.

Cell Treatment

All treatments were performed on a confluent monolayer of cells in a 100mm dish, with no media changes during the 5 days between subculture and treatment. Stock solutions of the appropriate chemicals were made up in DME medium and the pH was checked to confirm neutrality. The appropriate volume was then added directly to the 10ml of (5 day old) conditioned medium which covered the cells. This procedure was once again carried out in the laminar flow

hood. The cells were then returned to the 37°C incubator under an atmosphere of 95% air/5% CO_2 for five hours.

After this five hour exposure, the medium was removed by aspiration and the monolayer was washed twice with cold phosphate/saline. The cells were removed from the plates using a rubber-policeman, resuspended in phosphate/saline, and transferred to 5ml Falcon tubes. The cells were centrifuged at 50 revolutions per minute (RPM) for 5 minutes, the phosphate/saline was then removed and the pellet of cells was stored at -70°C until ornithine decarboxylase enzyme activity assay could be performed.

Optimizing Experimental Conditions

Preliminary experiments were undertaken to optimize the ornithine decarboxylase assay (chapter 3). It was verified that freeze thawing as apposed to sonication was the method of choice for disrupting cellular membranes and releasing ornithine decarboxylase protein. The assay procedure had best results when a saturating substrate concentration of 0.77μM was used. The reproducibility in the cellular response between days was optimized by achieving very low basal levels of ornithine decarboxylase, not using cells older than passage 22 and optimizing the chemical exposure time of the cells.

Assay for Ornithine Decarboxylase Activity

The cell pellet was thawed at room temperature and resuspended in assay buffer. The assay buffer was 25mM with respect to Tris-HCL (pH 7.5), 25μM with respect to pyridoxal 5'-phosphate, 2.5mM with respect to dithiothreitol, and 50μM with respect to EDTA. The cells were then disrupted by three successive

cycles of freezing and thawing, and the cellular debris was removed from the cellular extract by a 10 minute centrifugation at 12 x 10^3 RPM. The clear supernatant was removed and incubated for 10 minutes at 37°C.

The ornithine decarboxylase activity of the supernatant was then determined by using a prewarmed 37° center well assembly, designed for our purposes in our laboratory, which was used to measure the release of $^{14}CO_2$ from DL-[1-^{14}C]ornithine hydrochloride. The reaction was initiated by the introduction of 250μl of cellular extract into the center well which already contained L-ornithine (0.77μM final) (35).

Once initiated, the entire assembly was incubated in a 37°C water bath for 1 hour. The reaction was then terminated and CO_2 was simultaneously liberated from the center well by the injection of 500μl of 2M citric acid through the rubber stopper. CO_2 trapping time was refined by a series of preliminary studies (chapter 3) that indicated complete absorption of CO_2 by the ethanolamine:methoxyethanol (100μl; 2:1 v/v), was achieved at room temperature within 18 hours. Finally, the center well was removed and the outsides rinsed with 1ml ethanol (to ensure the complete absence of the ethanolamine:methoxyethanol from the outside of the center well). The center wells were then capped and discarded. Five ml of toluene-based scintillation fluid was added to the scintillation vial, i.e., the outer compartment, and mixed. The radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrophotometer which counted each tube for 10 minutes giving an average count per minute at the end of 10 minutes. The protein content of the cellular extract was determined by the bio-Rad procedure using bovine serum albumin as the protein standard (36).

Colony Forming Efficiency (CFE)

At approximately 4:30 pm the day before the experimental treatment, a stock flask of C3H 10T1/2 cells was trypsinized and seeded onto 60mm dishes at a density of 150 cells per plate. At approximately 9 am the following day the cells were exposed to the appropriate chemical for 5 hours. The medium was then removed, washed once with DME medium containing no fetal calf serum, and the cells were then resupplied with DME medium containing 10% fetal calf serum and allowed to grow at 37°C (95% air/ 5% CO_2) for 5 days. After this period of incubation the medium was removed and the plates were rinsed with distilled water. Methylene blue was then added to the plates and allowed to set for 10 to 30 minutes, depending on the freshness of the stain, and the cells were rinsed with distilled water. The colonies were counted under a dissecting microscope.

Statistical Analysis

One unit of ornithine decarboxylase activity, was defined as one pmole of CO_2 released per hour incubation per mg protein, and the "percent of control", defined as the percent change between treated and nontreated cells, were determined from raw $^{14}CO_2$ data. The error introduced by the ornithine decarboxylase assay procedure and by the variability among the triplicates performed on any one day were small compared to the variability in the ornithine decarboxylase response between days. The standard deviation was calculated by taking the square root of the sum of the relative differences (obtained for many different ODC activity values) squared, divided by the number of different ODC activities used minus one. The standard error of the mean was then calculated by dividing the standard deviation by the square root of n, where n is the

number of samples analysed in determining ornithine decarboxylase activity. Repeated plots of the gallate-induced ornithine decarboxylase response curve (which was replicated many times) showed that absolute variability was proportional to the magnitude of the reading, i.e., relative variability was constant. On this basis, standard error of the mean was determined to be 8.1% of the mean value. The error bars in the figures therefore represent 8.1% of the ornithine decarboxylase values plotted. Where the rates are expressed as a percentage of the mean value, the standard error of the values plotted were 2 x 8.1% or 16.2% of the mean.

RESULTS

The dose response curves for the various phenolics represent different concentration ranges because of constraints of solubility and cytotoxicity. All doses used were nontoxic to the cells (as determined by CFE). To standardize the condition of the cells before the administration of the test substances, we adhered rigidly to the protocol described in the material and methods section.

Phenolics which suppress the ornithine decarboxylase activity of C3H 10T1/2 cells

Quercetin was the most effective inhibitor of ornithine decarboxylase (figure 4.1). It decreased the enzyme activity in a dose-dependent manner, inhibiting maximally (48.1%) below control at 10μM. Further increases in quercetin concentrations to 50μM did not alter the response.

Ellagic acid was next in inhibitory effectiveness, decreasing ornithine decarboxylase activity in a dose-related fashion (figure 4.2). The maximum value of inhibition, 43% below control, occurred at 331μM. At still greater concentrations inhibition diminished until at 1660μM a 12% increase, above control, was reached.

Caffeic acid was the next most potent inhibitor of ornithine decarboxylase (figure 4.3). As with ellagic acid, the maximum inhibition for caffeic acid was obtained over a very broad concentration range, so that a 31.5% inhibition occurred at 16.5μM. Increasing the concentration of caffeic acid to 139μM did not further increase the inhibitory effect.

Eugenol was the least potent inhibitor of ornithine decarboxylase activity tested. Over a broad concentration range, 3-305μM the maximal inhibition ranged from 12 to 27.8% below control (figure 4.4).

Phenolics which enhance ornithine decarboxylase activity

Five hours of exposure of the C3H 10T1/2 cells to catechin caused a dramatic increase in the ornithine decarboxylase activity (figure 4.5). The response increased with increasing concentration and reached a maximum stimulation of 1502% to 2229% above control at 90μM to 900μM. Higher concentrations of this compound were cytotoxic, on the basis of colony forming efficiency.

Gallic acid induced a smooth dose-dependent increase in the activity of ornithine decarboxylase. A maximum stimulation of 400% was reached at 175μM (figure 4.6), and further stimulation did not occur with higher concentrations.

Catechol (figure 4.7) exhibited a dose response curve very similar to that of gallic acid, reaching a maximum stimulation of 350% at 300μM. At higher concentrations (up to 500μM) the response remained constant at 350%.

On the basis of a compound's ability to alter the activity of ODC from basal levels the order of potency of the tested compounds (from their greatest stimulatory to greatest inhibitory effect on ornithine decarboxylase activity in C3H 10T1/2 cells) is as follows: catechin >> gallic acid=catechol >> eugenol > caffeic acid > ellagic acid > quercetin (figure 4.8). As an alternative way of assessing potency, we compared concentrations of phenolics needed to double (in the case of the stimulatory phenolics) and to halve (in the case of the inhibitory

phenolics) the control levels of ornithine decarboxylase activity. Concentrations of 6.7μM catechin, 17.5μM gallic acid, or 40μM catechol increased control levels of ornithine decarboxylase by 100%, whereas 10μM quercetin or 331μM ellagic acid statistically decreased control levels of ornithine decarboxylase by 50%. The maximal effects of eugenol and caffeic acid did not reach 50% inhibition (figures 4.4 and 4.3 respectively), and thus the ED_{50} values are infinite. These two methods of assessing the effectiveness of the phenolics yield essentially the same order of effectiveness.

DISCUSSION

Phenolics exhibiting an inhibitory effect on ornithine decarboxylase activity

The observation that 40 minute preincubation with quercetin (10-30μM/mouse) inhibited (by 71%) the stimulation of ornithine decarboxylase activity by TPA (18), is consistent with the current findings that quercetin administration to the C3H 10T1/2 cells led to a decrease of up to 48% in the basal activity of ornithine decarboxylase. The 43% decrease in the activity of ornithine decarboxylase resulting from 331μM ellagic acid is also consistent with a previously observed 90% protection against benzo[a]pyrene induced mutagenesis in TA100 cells obtained by addition of 6μM ellagic acid to the culture medium (12,23).

The variable effects of ellagic acid on ornithine decarboxylase activity (13%-43% inhibition from 3 to 331μM and a stimulatory effect of up to 12% above control at 1660μM) indicate how profoundly the biochemical actions of some of these compounds may depend on their concentrations. Some of the conflicting reports of effects of the phenolics in the literature might be resolved if the researchers had carried out dose response studies instead of relying on single concentrations.

That 0.01-33.3μM caffeic acid induced a 10-31.5% decrease in ornithine decarboxlase activity is consistent with earlier findings that this compound also suppresses the mutagenicity of N'-methyl-N'-nitro-N-nitrosoguanidine (MNNG) in *salmonella typhimurium* strain TA1525 (at 4-10mg/ml) (13,14), and benzo[a]pyrene in TA100 (at 150μM) (12). It is also consistent with reports indicating that the presence of caffeic acid (2%) in the diets of mice leads to a 50% decrease in

the frequency of micronuclei induced by 7,12-dimethyl benz[a]anthracene (37), and with the evidence that caffeic acid (0.06mmol/g rat chow) is a weak inhibitor of the induction of tumors of the forestomach by benzo[a]pyrene (38). However it is not consistent with the finding that caffeic acid (1%) induces mild to moderate hyperplasia in the forestomach of Syrian golden hamsters (25). These conflicting results may again reflect differences in the concentrations used.

The 27.8% decrease in ornithine decarboxylase activity levels induced by 30.5μM eugenol is consistent with reports of a 50% inhibitory effect on benzo[a]pyrene mediated carcinogenicity at an application size of 10mg (26). However the finding is less consistent with results of Stich *et al.*, (1981) and Stich and Powrie (1982) who showed that the ability of eugenol to cause chromosomal aberrations in CHO cells is dose dependent. These conflicting results preclude simple correlation of the effects of eugenol *in vivo* with effects on ornithine decarboxylase *in vivo*.

Phenolics exhibiting a stimulatory effect on ornithine decarboxylase activity

The 2229% increase in ornithine decarboxylase activity induced by catechin in our study contrasts with its inhibitory effect on this same endpoint when combined with TPA (24). Gallic acid was somewhat less effective than catechin, increasing ornithine decarboxylase activity by 400% in the C3H 10T1/2 cells. This is in general agreement with the ability of 0.3mM gallic acid to cause chromosomal aberrations in CHO cells (2,15). Although low concentrations of gallic acid (2 to 110μM) did not alter the mutagenic effect of TA1525 exposed to a mixture of nitrite and methylurea, higher concentrations of gallic acid (2.1mM) inhibited mutagenesis by 50%. The 350% increase in ornithine

decarboxylase activity caused by administration of catechol (300μM) is in general agreement with the various types of chromosome aberrations reported for the administration of catechol to CHO cells (2,39,40).

As can be seen from table 4.2 a measure of agreement can be identified between the ability of a given phenolic to induce ornithine decarboxylase activity and its reported ability to act in a mutagenic, carcinogenic, or clastogenic nature. Similarly, a measure of agreement is also seen in the ability of a phenolic to suppress ornithine decarboxylase activity, to inhibit mutagenesis, carcinogenesis and cause no chromosomal aberrations.

Structure-activity relationships

A review of compounds structurally similar to those used in this study, with respect to mutagenicity, clastogenicity and mammalian carcinogenicity indicates that their biological actions depend upon features of their chemical structures (Table 4.2).

The abilities of related phenolics to inhibit a TPA induced increase in ornithine decarboxylase were slightly but significantly different in the order morin>fisetin=kaempferol>quercetin. Upon comparison of the structures of the tested phenolics small structural changes (compare catechin with quercetin) can result in very large alterations in the effects on ornithine decarboxylase activity. Substituents on the ring therefore determine the antioxidant or proxidant nature of a substance. The presence of meta hydroxyl groups on both rings was necessary for maximum inhibitory power, while ortho hydroxyl groups on one ring confer less inhibitory power. The similarities in the maximal inhibition of caffeic acid (31.5% inhibition) and eugenol (27.8% inhibition) may reflect similarities in their

structures (Table 4.1). Catechol and gallic acid were also similar in their effects on ornithine decarboxylase (350% and 400% stimulation). The observation that the reactivity gained (in terms of chromosomal aberrations) by introducing a third hydroxyl group onto the ring is lost by adding an additional carboxyl group (2), also holds in the current data for the induction of ornithine decarboxylase activity.

In summary then, dihydroxylated substances (specifically ortho more than meta), are slightly less effective than trihydroxylated phenols both in the induction of ornithine decarboxylase (current study) and in carcinogenic actions (25,26) as well as in the ability to induce chromosomal aberrations (2). The introduction of an alkyl group or replacment of a hydroxyl group by a methyl ester decrease the ability of a compound to induce ornithine decarboxylase.

The introduction of alkyl groups onto the ring increases lipophilicity. We therefore compared the compounds' abilities to increase or decrease ornithine decarboxylase activity with their hydrophobic character and their molecular weight. As can be seen in table 4.1, the effectiveness of a phenolic in producing its effect (whether stimulatory or inhibitory) increases with increasing molecular weight. In contrast, effects on ornithine decarboxylase activity of C3H 10T1/2 cells are independent of solubility.

We reported elsewhere (chapter 5) that the increase in ornithine decarboxylase activity by gallic acid is mediated by species resulting from the reduction of oxygen. These active species are not scavenged by superoxide dismutase or catalase, and resemble hydroxyl radicals in their ability to be scavenged by formate or benzoate. Transition metal ions are required for their

formation. On this basis it is relevent that the structural changes which increase the effectiveness in inducing ornithine decarboxylase, also increase the effectiveness of the substance as a proxidant. It is not a coincidence that the characteristics which make a given phenolic an effective inducer of ornithine decarboxylase (two unmethylated aromatic hydroxyls ortho- or para- to each other) are precisely what is needed to make it an effective reductant of oxygen. Conversely, characteristics which increase the antioxidant nature of a substance (for example an introduction of a ketone group), c.f. quercetin, makes it less effective as a reductant of oxygen, and less effective as an inducer of ornithine decarboxylase.

CONCLUSION

There is a correlation between the ability of phenolics to increase activity of cellular ornithine decarboxylase activity (as seen in the current study) and the previously reported mutagenic and clastogenic actions of the phenolics. Further studies of the effects of phenolics on mammalian systems with changes in ODC activity, papilloma formation, and clastogenic activity as well as correlations with pro- and anti-oxidant activity *in vitro* are needed. Conflicting reports of genotoxicity of some phenolics *in vivo* may be resolved when more appropriate comparisons are made using a wider range of concentrations, and detailed dose response relationships are available. Meanwhile, the response of ornithine decarboxylase to proxidants has promise as a screening procedure for their potential tumor promoting effects. Because small structural changes (compare catechin with quercetin) result in great changes in the effects on ornithine decarboxylase activity, a detailed study should be carried out using compounds specifically synthesized to test detailed and specific hypotheses regarding structure-function relationships.

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LEGENDS FOR FIGURES

FIGURE 4.1 Effect of quercetin on ornithine decarboxylase activity

Quercetin was added directly to a confluent monolayer of C3H 10T1/2 cells grown in Dulbecco's modified medium, supplemented with 10% (v/v) fetal bovine serum and 20mM HEPES. The cells were incubated for 5 hours at 37°C in 95% air/5% CO_2, in the presence of the phenolic, whereupon the medium was removed, the cells were scraped, pelleted and stored at -70°C until ornithine decarboxylase activity could be determined. Control values varied from one day to another from 46 to 101 pmole CO_2 released per hour per mg protein. The effects of the phenolic on ornithine decarboxylase, represented as the percent change from the control, induced by the treatment, i.e., ((ODC activity of treated cells - ODC activity of nontreated (control) cell)/ODC activity of nontreated cells) x 100. The error bars represent 8.1% of the ornithine decarboxylase value for any concentration considered. This value was calculated by repeatedly plotting the gallate-induced ornithine decarboxylase activity response curve ($n>>10$ for each point considered) and determining the relative difference.

FIGURE 4.2 Effect of ellagic acid on ornithine decarboxylase activity

The conditions were identical to those described for figure 1. The controls ranged from 43-98 pmole CO_2 released per hour per mg protein between days.

FIGURE 4.3 Effect of caffeic acid on ornithine decarboxylase activity

The conditions were identical to those described for figure 1. The controls ranged from 25-150 pmole CO_2 released per hour per mg protein between days.

FIGURE 4.4 Effect of eugenol on ornithine decarboxylase activity

The controls ranged from 25-55 pmole CO_2 released per hour per mg protein between days. The conditions were identical to those described for figure 1.

FIGURE 4.5 Effect of catechin on ornithine decarboxylase activity

The controls ranged from 106-168 pmole CO_2 released per hour per mg protein. The conditions were identical to those described for figure 1.

FIGURE 4.6 Effect of gallic acid on ornithine decarboxylase activity

The control ranged from 20-250 pmole CO_2 released per hour per mg protein. The conditions were identical to those described for figure 1.

FIGURE 4.7 Effect of catechol on ornithine decarboxylase activity

The controls ranged from 98-152 pmole CO_2 released per hour per mg protein. The conditions were identical to those described for figure 1.

FIGURE 4.8 Maximal effects of phenolics on ornithine decarboxylase activity

The maximum percent change from control (produced at the most effective concentration) is shown. "Max dose" means the dose of phenolic in μM which gave the most extreme response. "Max percent change" refers to the maximum percent change between treated and nontreated cells occurring at the maximum dose (calculated as described in figure 4.1). "CN" represents catechin; "GA" gallic acid; "CL" catechol; "EUG" eugenol; "CA" caffeic acid; "EA" ellagic acid; and "QUE" represents quercetin.

TABLE 4.1 Physical and experimental data on the tested phenolics

This table summarizes data derived from figures 4.1 through 4.7. "DOSE AT MAX EFFECT" refers to the dose of the particular phenolic which caused the maximal effect (be it positive or negative) on ornithine decarboxylase activity.

"MAX EFFECT IN (pmolCO_2/hr/mg protein)" refers to the actual enzyme activity measured at the most effective dose, where "C" indicates control values i.e., no treatment, and "P" indicates the actual enzyme activity after treatment. "MAX % change from control" refers to the percentage difference between control and treated values. The + indicates that the treatment was stimulatory over control and the - indicates that the treatment was inhibitory with respect to control.

TABLE 4.2 Phenolics and their effects on ODC activity and their reported effects in other systems

The effects of the phenolics on the activity of ODC refers to the percent change from control caused by a 5 hour incubation of the phenolic with C3H 10T1/2 cells. The mutagenic effect refers to results from the *Salmonella typhimurium* test. Carcinogenic data were obtained from mammalian *in vivo* studies and chromosomal aberration data were obtained using the chinese hamster ovary (CHO) cell system.

FIGURE 4.1

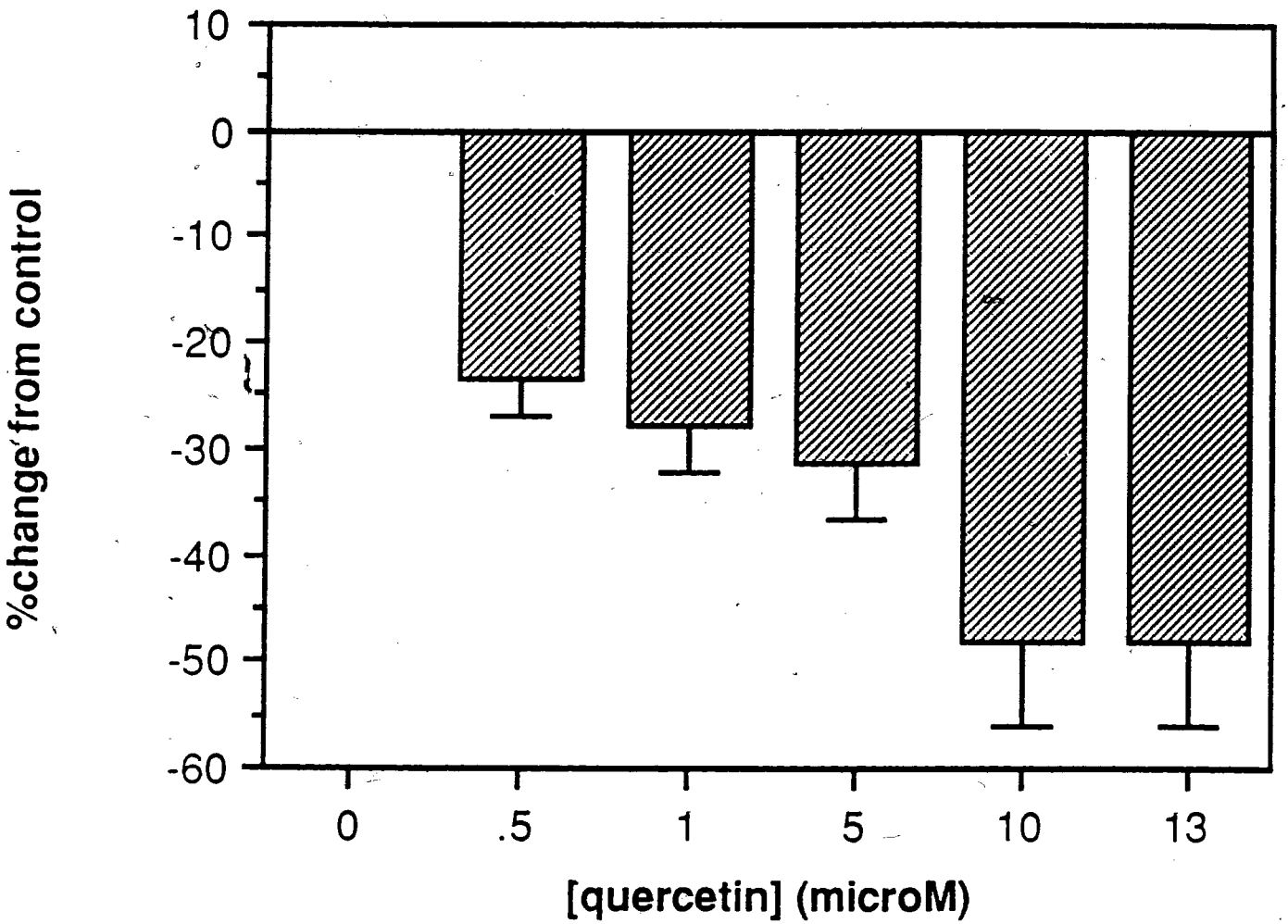

10
0
-10
-20
-30
-40
-50
-60
%change from control
0
.5
1
5
10
13
[quercetin] (microM)

FIGURE 4.2

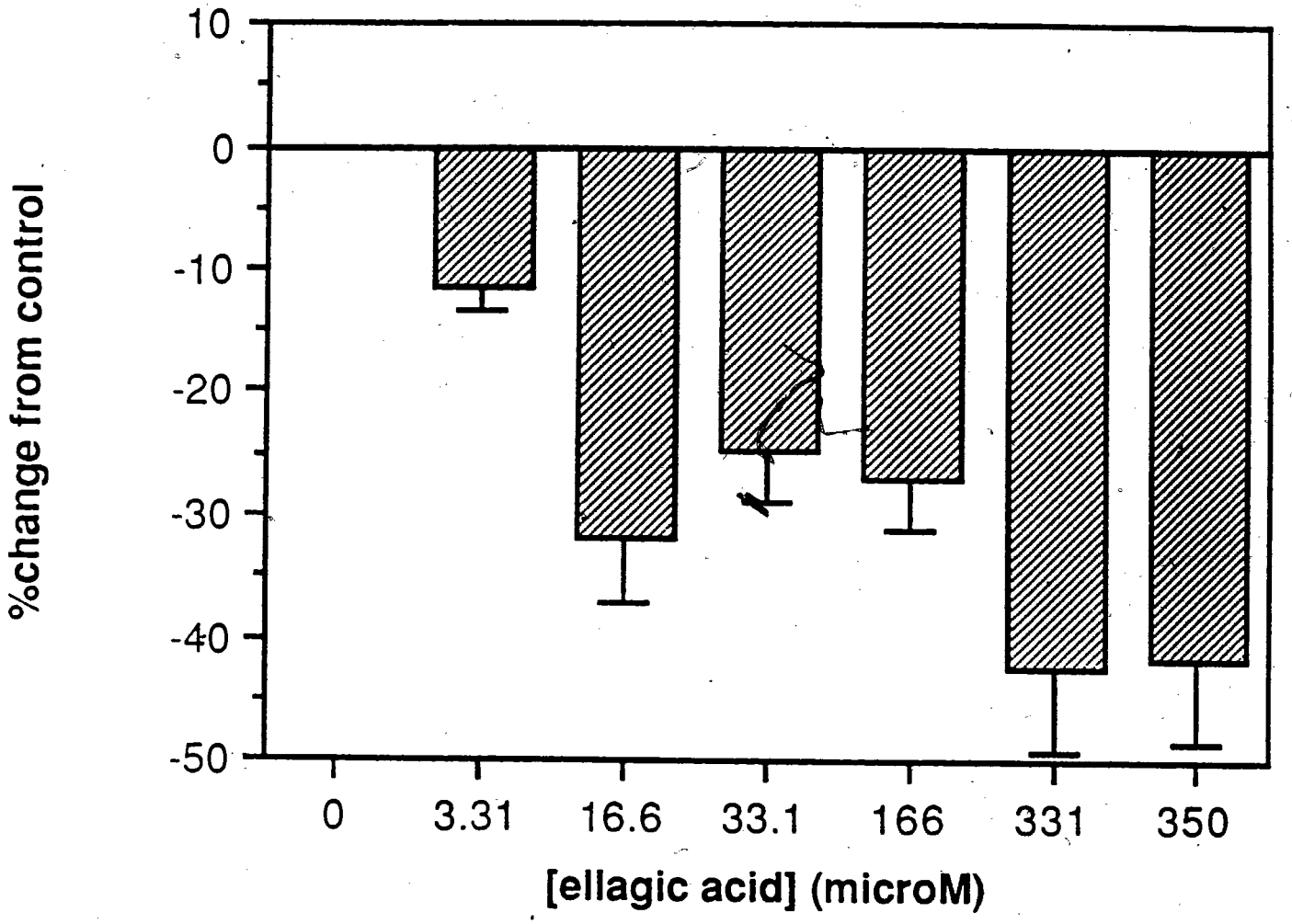

10
0
-10
-20
-30
-40
-50
%change from control
0
3.31
16.6
33.1
166
331
350
[ellagic acid] (microM)

FIGURE 4.3

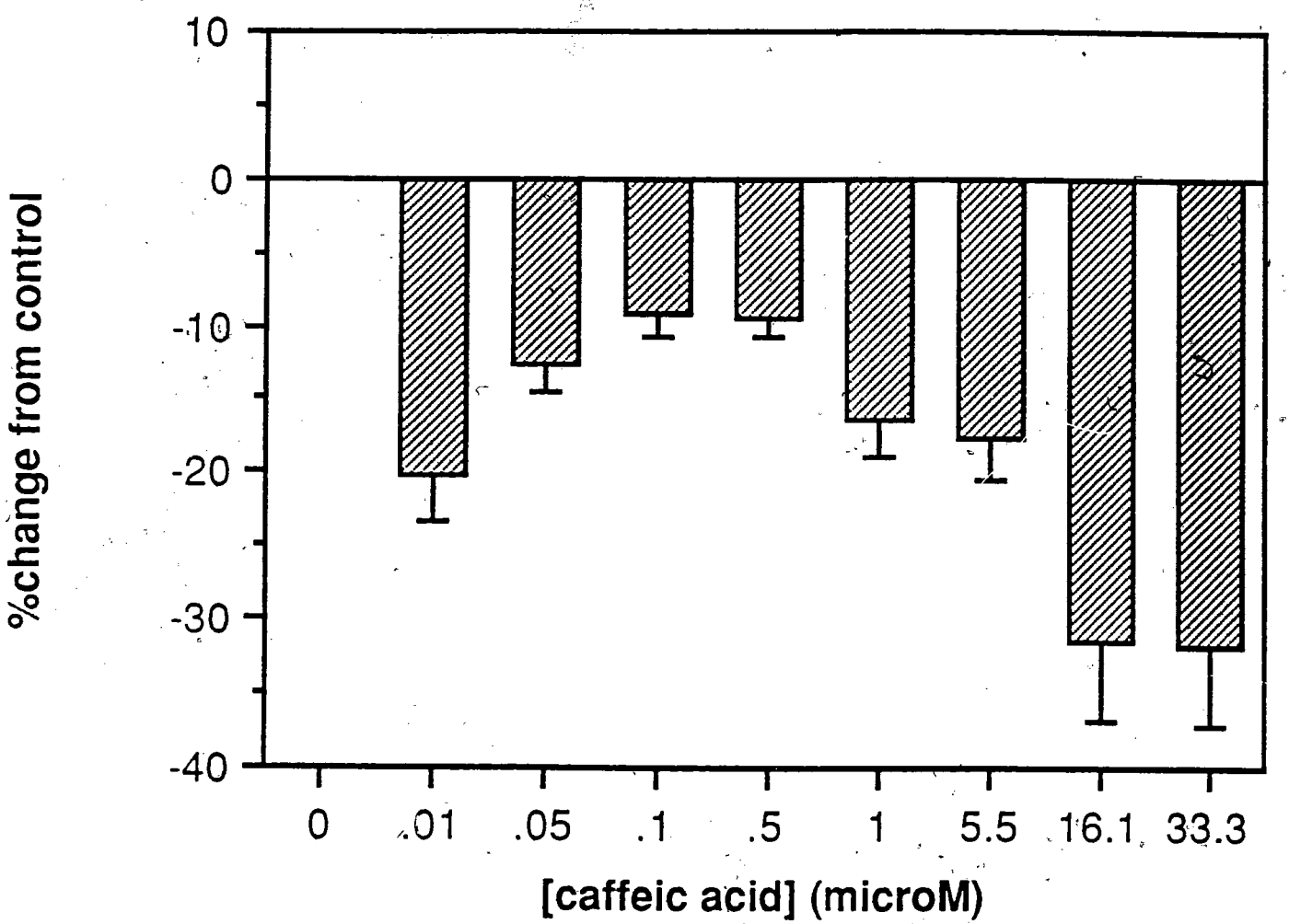

10
0
-10
-20
-30
-40
%change from control
0
.01
.05
.1
.5
1
5.5
16.1
33.3
[caffeic acid] (microM)

FIGURE 4.4

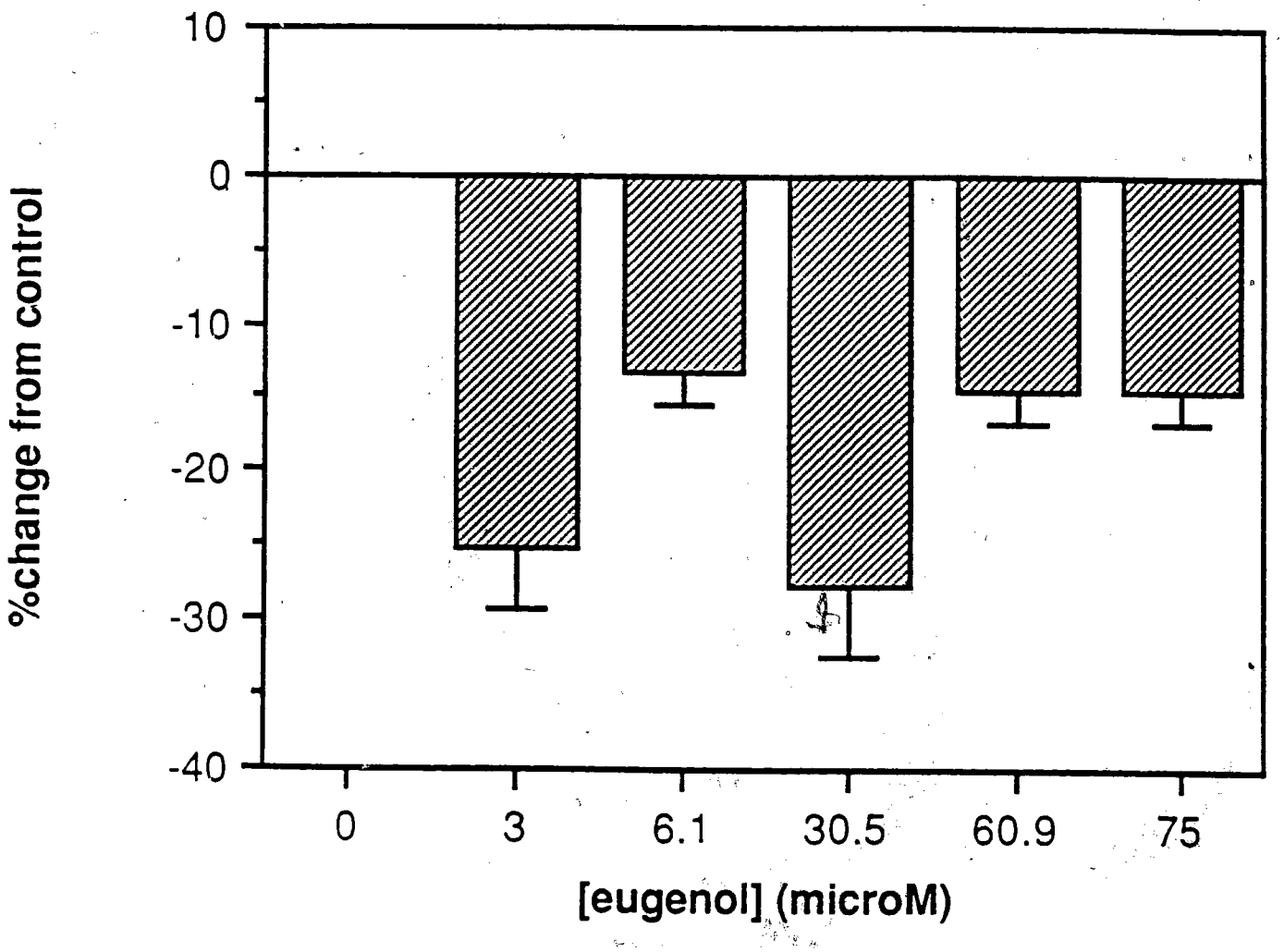

10
0
-10
-20
-30
-40
%change from control
0
3
6.1
30.5
60.9
75
[eugenol] (microM)

FIGURE 4.5

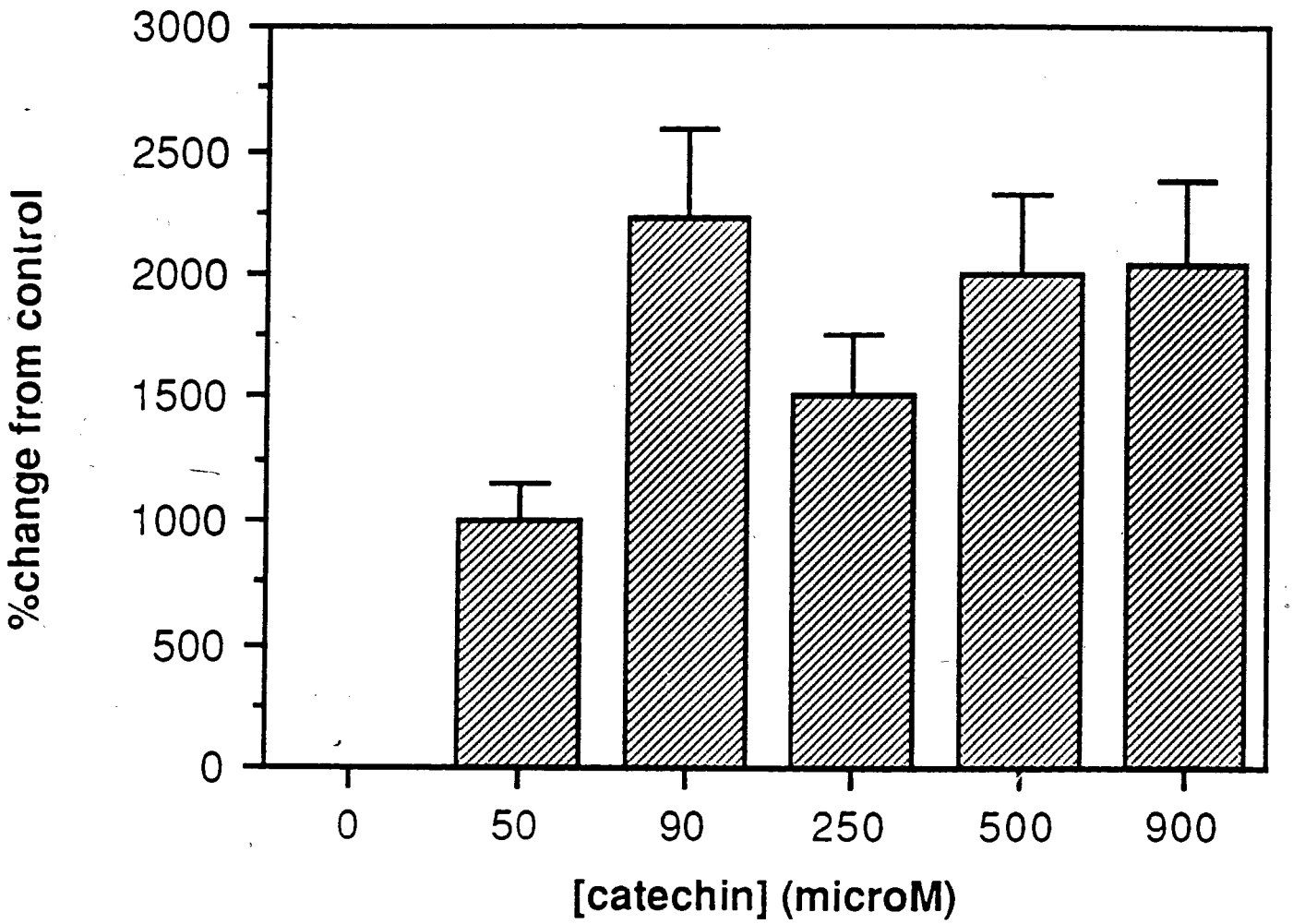

3000
2500
2000
1500
1000
500
0
%change from control
0
50
90
250
500
900
[catechin] (microM)

FIGURE 4.6

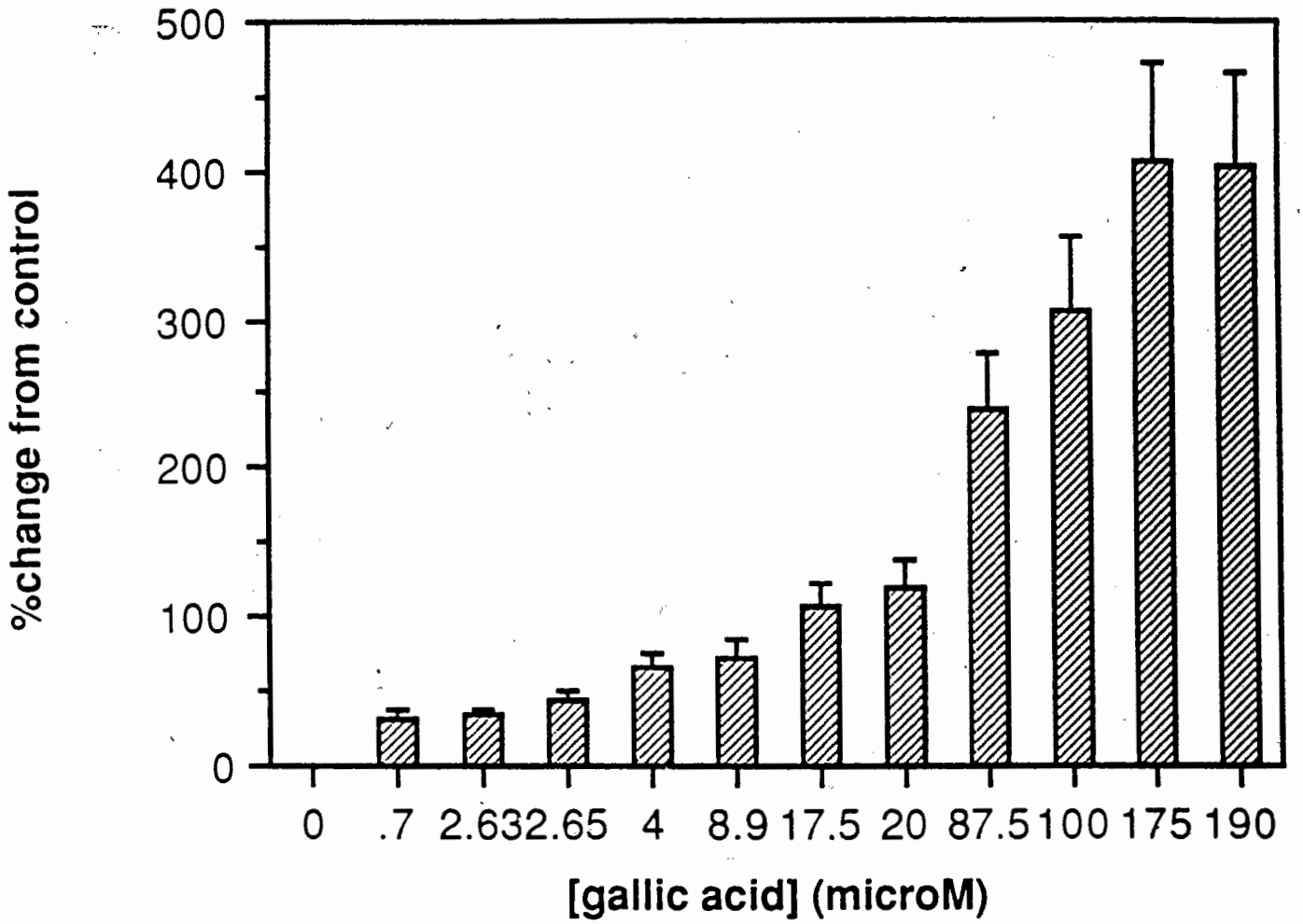

500
400
300
200
100
0
%change from control
0
.7
2.63
2.65
4
8.9
17.5
20
87.5
100
175
190
[gallic acid] (microM)

FIGURE 4.7

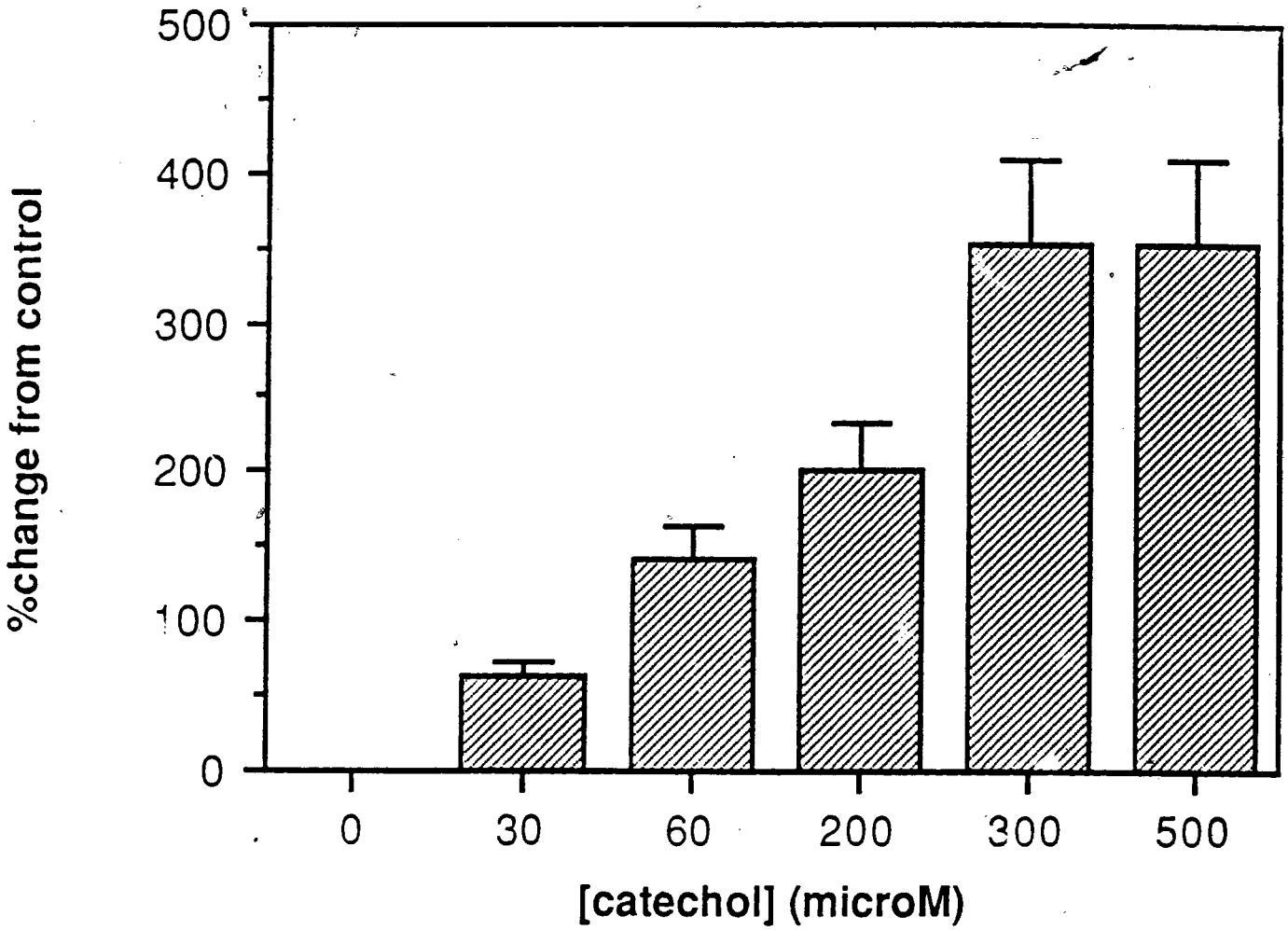

%change from control
500
400
300
200
100
0
0
30
60
200
300
500
[catechol] (microM)

FIGURE 4.8

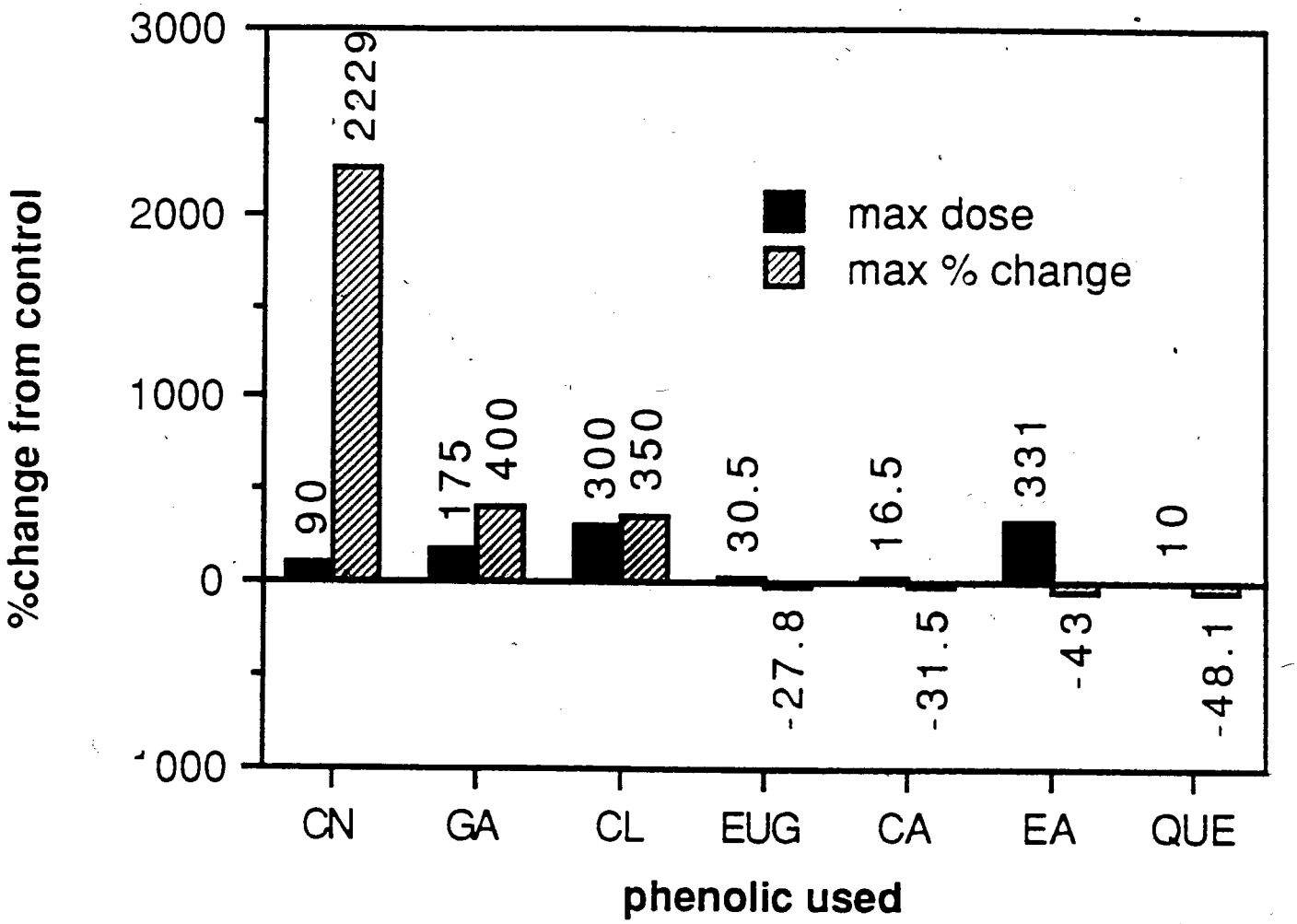
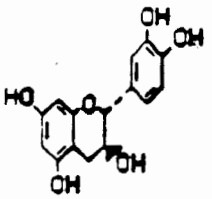
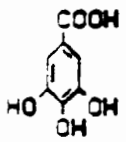
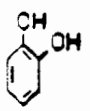
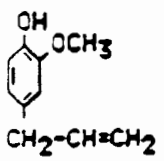
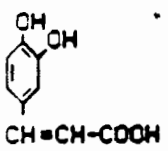
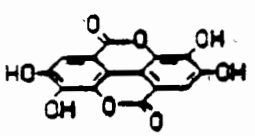
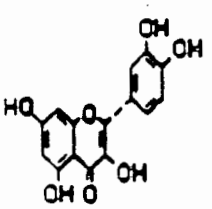

3000
2000
1000
0
000
%change from control
max dose
max % change
90
2229
175
400
300
350
30.5
-27.8
16.5
-31.5
331
-43
10
-48.1
CN
GA
CL
EUG
CA
EA
QUE
phenolic used

TABLE 4.1

COMPOUND	STRUCTURE	DOSE AT MAX EFFECT	MAX EFFECT IN (pmolCO2/hr/ mg protein)	MAX % change from control
catechin (mw 290.28)		90uM +	C=117.93 P=2713	+2200%
gallic acid (mw 170.12)		175uM+	C=64.68 P=304	+400%
catechol (mw 110.11)		300uM+	C=101 P=455	+350%
eugenol (mw 164.20)		30.5uM	C=22.40 P=16.2	-27.8%
caffeic acid (mw 180.15)		16.6uM	C=135.01 P=92.5	-31.5%
ellagic acid (mw 302.19)		331uM	C=42.99 P=29.45	-42%
quercetin (mw 302.23)		10uM	C=47.4 P=24.6	-48.1%

C = pmol CO_2 released/hour/mg protein in control cells i.e., no treatment
P = pmol CO_2 released/hour/mg protein in treated cells

Table 4.2

Compound	number of hydroxyl groups	effect on ODC (current study)	mutagenic	carcinogenic	chromosomal aberrations
catechin	5	+2200%	inhibit (1mM)	no data	no data
gallic acid	3	+400%	no effect (2-110uM) suppressed (2.1mM)	no data	positive (0.05mg/ml)
catechol	2	+350%	enhanced	yes	positive (0.05mg/ml)
eugenol	1	-27.8%	no data	no	no effect (0.05mg/ml) positive (0.4mg/ml)
caffeic acid	2	-31.5%	suppressed	both effects reported	both effects reported
ellagic acid	4	-42%	suppressed	no data	no data
quercetin	5	-48.1%	no data	no	no effect

CHAPTER V

ACTIVE OXYGEN AND METALS PLAY A ROLE IN THE STIMULATORY EFFECT OF GALLATE ON THE ACTIVITY OF ORNITHINE DECARBOXYLASE

ABSTRACT

Some scavengers of activated oxygen enhanced, while others moderated the induction of ornithine decarboxylase (ODC) in the presence of gallate. In the absence of gallate, none of the scavengers had any significant effects. Thus, superoxide dismutase (10 or 50U/ml) enhanced the gallate-induced stimulation of ODC by 60 or 215%, catalase at 50U/ml increased the response by up to 42%, and mannitol (50mM) stimulated by up to 200%. In contrast, formate (50 or 75mM), benzoate (50 or 75mM) or desferrioxamine (50 or 100μM) prevented the gallate-induced increase in ODC activity. These inhibitory effects of formic acid, benzoate and desferrioxamine went beyond merely preventing the ODC response to gallate. They transformed gallate from a stimulator of ODC to a suppressor of ODC, levels falling to 50 to 80% below *control* at almost all concentrations of gallate. Comparing the current results in cultured cells, with previous data for the *in vitro* autoxidation of gallate, compounds which accelerated the autoxidation, in general increased the ODC activity, while compounds which slowed the autoxidation also decreased ODC activity. Thus, some product generated in the autoxidation of gallate (and not gallate itself) is responsible for the increase in the ODC activity. The failure of superoxide dismutase or catalase to inhibit, suggests that the ODC stimulation is not mediated by gallate-generated superoxide or H_2O_2. That gallate in the presence of formate and benzoate decreased ODC to below basal levels, indicates that some species related to the hydroxyl radical

may mediate the induction of ODC activity in the presence of gallate, and that gallate itself is a suppressor of ODC activity.

INTRODUCTION

Carcinogenesis is a multistep process, with distinguishable initiation and promotional phases. In mouse skin, initiation requires a single subthreshold application of a carcinogen (1,2) which leads to permanent genetic changes (3,4). This event, however, does not give rise to tumors unless the initiated skin is repeatedly exposed to low doses of a promoter, upon which skin papillomas appear after a 6-10 week latency period (5). This promotional phase, is initially reversible, but becomes irreversible with prolonged exposure.

Many tumor promoters are also free radical-generating compounds. Benzoyl peroxide, lauroyl peroxide and the xanthine/xanthine oxidase system, to name only a few, are tumor promoters (1,4,6,7). Phorbol esters, such as 12-O-tetradecanoylphorbol-13-acetate (TPA), which are the most potent promoters (8), may act through free radical intermediates (9,10). The role of active oxygen is inferred from a decrease in the effectiveness of promoters in the presence of many antioxidants, including superoxide dismutase or catalase. Thus antioxidants like superoxide dismutase or catalase decrease the number of papillomas produced in mice by TPA, or decrease the number of transformed foci in cell culture (6,7,11,12,13,14).

TPA is the most widely used promoter. Although its mode of action is not entirely clear, it causes many biochemical changes (8,15,16,17). Among these, the induction of ornithine decarboxylase activity, epidermal cell proliferation, and the appearance of dark basal keratinocytes have the best correlation with its promoting ability (18,19,20,21,22). Superoxide dismutase in combination with catalase, or mannitol acting alone, inhibit the induction of ornithine decarboxylase

by TPA (23), as do antioxidants such as butylated hydroxyanisole and retinoic acid (24,25,26,27,28). This suggests that free radical producing systems may act at the promotional stage, via an increase in the ornithine decarboxylase activity.

There is a relationship between the ability of a phenolic to increase or decrease ornithine decarboxylase activity and its reported ability to enhance or inhibit tumor promotion (chapter 4). For example, oral administration of catechol causes epidermal damage and consequent regenerative hyperplasia in the pyloric region of Syrian golden hamsters (29) and external application enhances the carcinogenicity of benzo[a]pyrene in mouse epidermis (30). Moreover, addition of catechol to the culture medium also increases ornithine decarboxylase activity in mouse embryo C3H 10T1/2 fibroblast cells (chapter 4). TPA and teleocidin induction of papillomas when topically applied to the skin of mice can be decreased by the prior application of quercetin (31,32) a compound which also decreases the activity of ornithine decarboxylase in C3H 10T1/2 cells (chapter 4).

Many dietary and environmental phenolics produce free radicals by autoxidation. For the current study we selected one of the phenolics, namely gallic acid (gallate), to determine the extent to which its stimulatory effect on ornithine decarboxylase activity could be supressed by the prior presence of a metal chelating agent or scavengers of active oxygen. A major reason for the selection of gallate was the availability of a body of data (appendix) on the effects of superoxide dismutase, catalase, benzoate and desferrioxamine on the rates of oxidation of gallate.

MATERIALS AND METHODS

Reagents

DL-[1-^{14}C] Ornithine (61 mCi/mmol) was obtained from New England Nuclear. 12-O-tetradecanoylphorbol-13-acetate (TPA) lot #34F-0682, pyridoxal 5'-phosphate, L-ornithine-HCL, disodium ethylenediaminetetracetate (EDTA), citric acid monohydrate, bovine serum albumin lot #25F-0057, superoxide dismutase EC 1.15.1.1, bovine blood, 3100U/mg protein lot #93F-9305, catalase EC 1.11.1.6, bovine liver, 11000U/mg protein lot #82F-0838, formic acid (sodium salt), benzoic acid (sodium salt), dithiothreitol, and D-mannitol were from Sigma Chemical Co., St. Louis, Mo. Tris(hydroxymethyl)methylamine, and 2-methoxyethanol, were from BDH Chemicals, Vancouver, B.C. Ethanolamine was from Fisher Scientific, Fair Lawn, N.J. Gallic acid [3,4,5-trihydroxybenzoic acid] was from Aldrich Chemical Co., Milwaukee, Wis. Bradford protein assay dye lot #31751 was from Bio-Rad Laboratories, Richmond, Calif. Desferrioxamine (Desferal mesylate) was a gift from CIBA Pharmaceutical Co., Summit, N.J.

Cell Line

The contact sensitive mouse embryo cell line, C3H 10T1/2, passage 8, was obtained from American Type Culture Collection, Rockville, Maryland. Cells for experimental purposes, i.e., treatment, were grown in Nunclon 100mm plastic dishes in Dulbecco's modified Eagle's medium ("growth medium") obtained from Gibco Laboratories Grand Isle., New York. This was supplemented to 10% (v/v) fetal bovine serum (FCS), of "select silver" grade, from Flow Laboratories, McLean Virginia, and 20mM HEPES [N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid], from Sigma Chemical Co.

Stock cultures were maintained in 80cm² Nunclon flasks. Both the 80cm² flasks and the 100mm dishes were seeded at the same density (approximately 120 x 10^4 cells/plate) and allowed to reach confluency over five days, with no change of growth medium. The cells were maintained in humidified air with 5% CO_2 at 37°C.

Trypsinization procedures for this particular cell line were optimized, and all subsequent trypsinizations were then carried out systematically; cell medium was poured off and the cells were washed once with 0.05% trypsin (porcine parvovirus tested, lot #72P4615 obtained from Gibco) dissolved in phosphate buffered saline (0.8% NaCl; 0.02% KCl; 0.115% Na_2HPO_4; 0.02% KH_2PO_4; 0.02% EDTA). The cells were then incubated with 0.05% trypsin in phosphate buffered saline for 30 seconds and the trypsin poured off; residual trypsin was allowed to stay on the cells for an additional 45 seconds. The cells were then resuspended in growth medium and the cell density was determined using a Bright-line hemacytometer. This entire procedure was performed under sterile conditions in a laminar flow hood.

Cell Treatment

All treatments were performed on a confluent monolayer of cells in a 100mm dish, with no media changes during the 5 days between subculture and treatment. Stock solutions of gallic acid were made up in double distilled water. Stock solutions of the appropriate scavengers were also made up in double distilled water. Stock solutions of enyzymes were kept at 0°C. The appropriate amount of scavenger was added directly to the 5 day old conditioned medium (10mls) and cells. The cells were incubated at 37°C for one hour before the

addition of gallate.

After the predetermined exposure period, the medium was removed by aspiration and the monolayer was washed twice with cold phosphate buffered saline. The cells were removed from the plates and resuspended in phosphate buffered saline, by scraping with a rubber policeman, and the cell suspension was transferred to 5ml Falcon tubes. The tubes were then centrifuged at 50 revolutions per minute (RPM) for 5 minutes, and the phosphate/saline was removed. The pellet of cells was stored at -70°C until the ornithine decarboxylase enzyme assay could be performed.

Optimizing experimental conditions

In a series of experiments a number of experimental parameters were optimized before the ornithine decarboxylase assay was performed (chapter 3). The results of these experiments identified the best method for disrupting the cellular membranes, saturating substrate concentration for the ODC assay, maximal CO_2 trapping time, optimal cellular conditions, preferred medium for chemical stock solutions and optimal exposure time for the cells.

Ornithine Decarboxylase Activity Assay

Ornithine decarboxylase activity was determined by the $^{14}CO_2$ trapping method described in chapter 4. Cellular supernatant from the various chemical treatments was mixed, with DL-[1-$^{14}CO_2$] ornithine hydrochloride. in the center tube of the CO_2 trapping assembly (33). The reaction progressed at 37°C for one hour and the $^{14}CO_2$ liberated was collected in the main chamber by 2:1 ethanolamine:methoxyethanol. After complete $^{14}CO_2$ liberations the amount

absorbed was determined by a Packard Tri-Carb liquid scintillation spectrophotometer. The protein content of the cellular extract was determined by the bio-Rad procedure using bovine serum albumin as the protein standard (34).

Colony Forming Efficiency (CFE)

Starting at approximately 4:30 pm the day before the experimental treatment, a stock flask of C3H 10T1/2 cells was trypsinized and seeded on 60mm dishes at a density of 150 cells per plate. At approximately 9 am the following day the cells were exposed to the appropriate chemical for 5 hours. The medium was then removed, washed once with DME medium containing no fetal calf serum, and the cells were then resupplied with DME medium containing 10% fetal calf serum and allowed to grow at 37°C (95% air/5% CO_2) for 5 days. The medium was then removed and the plates were rinsed with distilled water. The cells were fixed and stained with 0.1% methlene blue in 50% methanol for at least 10 minutes. The cells were rinsed with distilled water and allowed to air dry. The colonies were counted under a dissecting microscope.

Statistical Analysis

One unit of ornithine decarboxylase activity, was defined as one pmole of CO_2 released per hour incubation per mg protein. The "percent change from control", is the percent change in $^{14}CO_2$ release between the ornithine decarboxylase activity of treated and nontreated cells caused by the treatment.

Percent change equals ((treated cells - nontreated cells)/ nontreated cells) x 100. The error introduced by the ornithine decarboxylase assay procedure and by the variability among the triplicates performed on any one day were small

compared to the variability in the ornithine decarboxylase response between days. The standard deviation was calculated by taking the square root of the sum of the relative differences (obtained for many different ornithine decarboxylase activity values) squared, divided by the number of different ornithine decarboxylase activities used minus one. The standard error of the mean was then calculated by dividing the standard deviation by the square root of n, where n is the number of samples analysed in determining ornithine decarboxylase activity. Repeated plots of the gallate-induced ornithine decarboxylase response curve (which was replicated many times) showed that absolute variability was proportional to the magnitude of the reading, i.e., relative variability was constant. On this basis, standard error of the mean was 8.1% of the mean. The error bars in the figures therefore represent 8.1% of the ornithine decarboxylase values plotted. Where the rates are expressed as a percentage of the mean value, the standard error of the values plotted were 2 x 8.1% or 16.2% of the mean.

RESULTS

In the absence of gallate, none of the scavengers tested, (mannitol, catalase, superoxide, dismutase, formate, benzoate and desferrioxamine) significantly altered the basal levels of ornithine decarboxylase (figures 5.1-5.7). However, they altered the gallate-induced stimulation of ornithine decarboxylase, under at least some of the conditions. Three of the scavengers (superoxide dismutase, catalase or mannitol) enhanced the induction of ornithine decarboxylase by gallate, refuting the hypothesis that they might ameliorate the actions of gallate on ornithine decarboxylase activity by scavenging radical intermediates. The other three compounds tested (formate, benzoate or desferrioxamine) partially or completely prevented the stimulatory effect of gallate on ornithine decarboxylase activity. In fact, their presence caused gallate to become an inhibitor of ornithine decarboxylase activity, since in their presence ornithine decarboxylase activity was decreased to significantly below basal levels.

Compounds that enhance the gallate induced ornithine decarboxylase activity

Mannitol (25 and 50mM) did not significantly effect the stimulation of ornithine decarboxylase activity induced by 8.9-89.5μM gallate. However, at 175μM gallate, mannitol (at either 25 or 50mM) enhanced the stimulatory effect on ornithine decarboxylase activity by about 200% (figure 5.1).

Catalase at 10U/ml did not effect the gallate induced ornithine decarboxylase activity (figure 5.2) but 50U/ml catalase increased the activity of ornithine decarboxylase by 32, 25, 42 and 40% at gallate concentrations of 8.9, 17.5, 89.5 and 175μM respectively.

Superoxide dismutase augmented the stimulatory effects of gallate (figure 5.3), the effect being significantly greater at 50U/ml superoxide dismutase than at 10U/ml. The effects of 10U/ml superoxide dismutase were not statistically significant at gallate concentrations of 8.9 and 17.5μM. At 89.5 and 175μM gallate, 10U/ml SOD increased gallate induced ornithine decarboxylase activity by 45% and 60%. At 50U/ml however, superoxide dismutase augmented the gallate induced ornithine decarboxylase activity by, 35%, 30%, 117% and 215% at gallate concentrations of 8.9, 17.5, 89.5 and 175μM, respectively.

Compounds which inhibit the effects of gallate on ornithine decarboxylase activity

In contrast to the abovementioned compounds, formate totally prevented the stimulatory effects of gallate (figure 5.4). In the presence of gallate formate decreased ornithine decarboxylase below control values, from 50% below controls at 8.9μM gallate to 74% below control at 175μM gallate. The inhibitory response was maximal at 50mM formate, there being no increase in the effect when the concentration of formate was increased to 75mM.

Benzoate was as inhibitory as formate at low gallate concentrations, but it was somewhat less inhibitory at the highest concentrations of gallate. Thus the greatest inhibitory effect of benzoate (seen at 8.9μM gallate) being a 65% decrease in ornithine decarboxylase activity below control) (figure 5.5). The degree of inhibition decreased progressively as the concentration of gallate increased, until at 175μM gallate no effects of benzoate on gallate induced ornithine decarboxylase activity were observed.

The effects of desferrioxamine were similar to those of benzoate (figure 5.6), in that at the highest concentration of gallate the stimulatory effects of

gallate began to overpower the inhibitory action of desferrioxamine. The effects thus ranged from 63% below control at 8.9μM, to 80% above control at 175μM (compared with a 314% increase above control caused by gallate alone).

Stimulation of ornithine decarboxylase activity involves de novo protein synthesis

We made several attempts to determine whether the stimulation of ornithine decarboxylase activity by gallate involved induction of *de novo* synthesis of either mRNA or protein. We made two attempts to isolate newly synthesized ornithine decarboxylase mRNA, but failed to produce nondegraded mRNA. We also carried out three incubations of C3H 10T1/2 cells with gallate plus actinomycin D, and three studies in which the cells were preincubated with actinomycin D, with varied and inconclusive results. However cycloheximide produced a dramatic, consistent, and statistically significant, inhibition of the ability of gallate to stimulate ornithine decarboxylase. In six separate experiments, three involving preincubation and three co-incubation of C3H 10T1/2 cells with cycloheximide (5μg/ml or 10μg/ml), cycloheximide caused a consistent 80-100% inhibition of the expected gallate-induced increase in ornithine decarboxylase activity. In four out of the six experiments the inhibition was total (figure 5.7).

On the basis that the effects of cycloheximide are primarily due to its inhibition of RNA translation, one can conclude that the increase in ornithine decarboxylase activity reflects *de novo* protein synthesis. However, because of the inconclusive studies with actinomycin D and mRNA synthesis, it remains to be determined whether the control is exerted at the level of DNA transcription or mRNA translation.

Comparison of the effects of scavengers on the in vitro autoxidation of gallate and the gallate-induced increase in ornithine decarboxylase activity

Comparing the effects of scavengers on the gallate-induced increase in ornithine decarboxylase activity with previous studies of their effects on the *in vitro* autoxidation of gallate a few noteworthy relationships are identified (table 5.1). In the case of three scavengers of the four for which we have data, an increase or decrease in autoxidation rate was associated with an ornithine decarboxylase activity response in the same direction. Two of the three scavengers which accelerate autoxidation also stimulated ornithine decarboxylase activity, while one scavenger/chelator which slowed autoxidation diminished the ornithine decarboxylase activity response. The one exception was benzoate which stimulated quinonoid product formation but diminished the ornithine decarboxylase activity response. This single observation proves that it is not the quinonoid product which stimulates ornithine decarboxylase activity.

DISCUSSION

Role of H_2O_2 and O_2^- in the gallate-induced increase in ornithine decarboxylase activity

The stimulatory effects of superoxide dismutase, catalase or mannitol on the gallate-induced increase in ornithine decarboxylase activity contrast with their inhibitory actions on TPA-induced increases in ornithine decarboxylase activity (23). The results suggest that superoxide and H_2O_2 are involved in the induction of ornithine decarboxylase activity by TPA, but not gallate. Autoxidation of gallate is somewhat accelerated upon the removal of superoxide and H_2O_2 (refer to table 5.1 and appendix).

The lack of effect of 10U/ml catalase and the small effect seen at 50U/ml catalase shows that accumulation of hydrogen peroxide is not important to the stimulation of ornithine decarboxylase. The small stimulatory effect of catalase may in part be due to the previously observed small increase in the rate of autoxidation of gallate by product removal (see appendix), and in part to the increase in O_2 tension, resulting from O_2 produced in the enzymatic decomposition of hydrogen peroxide. That the presence of superoxide dismutase stimulates rather than inhibits the induction of ornithine decarboxylase activity by gallate suggests that superoxide did not mediate the stimulation of ornithine decarboxylase activity by gallate. Instead it is more plausible that superoxide dismutase stimulates by accelerating autoxidation of gallate.

The abovementioned data taken together suggest that ornithine decarboxylase activity is induced by some active oxygen species (other than superoxide or H_2O_2) produced during the autoxidation of gallate. For example, superoxide dismutase

certainly enhances production of H_2O_2, (in competition with other products of the reactions of superoxide). However the stimulatory effects of catalase exclude the possibility that ornithine decarboxylase stimulation is mediated by hydrogen peroxide. More plausibly, the presence of superoxide dismutase increases steady state levels of other active species previously removed by reaction with superoxide (35). As a specific example, in a related reaction reduction of molecular oxygen by 3-hydroxyanthranilic acid, superoxide dismutase enhances the yield of hydroxyl radicals (36).

Role of metal ions in the gallate-induced increase in ornithine decarboxylase activity

The effects of desferrioxamine are consistent with a requirement for redox cycling of metals in the autoxidation of gallate, reflected in the dramatic decreases in the rate of autoxidation of gallate caused by desferrioxamine (see appendix). The inhibitory actions of desferrioxamine therefore reinforce the view that some intermediate product of the autoxidation of gallate is responsible for stimulation of ornithine decarboxylase. At high concentrations of gallate, sufficient quantities of the intermediate are produced (despite the presence of desferrioxamine) to cause substantial stimulation of ornithine decarboxylase (figure 5.6 last two bars).

Role of the hydroxyl radical in the gallate-induced increase in ornithine decarboxylase activity

The strong inhibitory effects of the two hydroxyl radical scavengers (formate and benzoate) suggest that the stimulatory intermediate may be hydroxyl radicals. This is reinforced by the effect of benzoate on the rate of autoxidation of gallate (table 5.1). Since benzoate increases the rate of autoxidation of gallate

but decreases the gallate-induced increase in ornithine decarboxylase activity, strongly suggests it must be the hydroxyl radical and not the quinonoid product which is necessary for the stimulation of ornithine decarboxylase activity. The similar effects of the hydroxyl radical scavengers benzoate and formate and the metal chelator desferrioxamine suggests that hydroxyl radicals formed in a Fenton-type reaction may be responsible for the stimulation. However the ineffectiveness of mannitol, another hydroxyl scavenger, on ornithine decarboxylase activity introduces a margin of doubt in this conclusion. Either the mannitol has less access to the (intracellular) site of hydroxyl radical production, or the radicals are generated in a "site specific" manner accessible to formate or benzoate, but not to mannitol (37). Based on the dramatic interference of cycloheximide on the gallate induced increase in ornithine decarboxylase activity, the simplest conclusion is that the radicals responsible act to stimulate new protein synthesis. Future studies should consider the possibility that cycloheximide acts by other mechanisms in addition to simple prevention of mRNA translation into protein. More detailed studies of the nature and cellular sites of action of bioactive phenolics will yield significant advances in the mechanisms of tumor promotion.

CONCLUSION

The effects of free radical scavengers on the gallate induced increase in ornithine decarboxylase activity parallel the effects of scavengers on the rate of reduction of oxygen by gallate *in vitro* (see appendix). Inhibitory effects of hydroxyl radical scavengers suggest that the hydroxyl radicals are involved in the induction of ornithine decarboxylase activity by gallate. The roles of active oxygen in tumor promotion and the abilities of tumor promoters to induce ornithine decarboxylase suggest a relationship between the production of oxygen radicals and both tumor promotion and induction of ornithine decarboxylase activity. Moreover, the effectiveness of scavengers in modulating the production of oxygen radicals induced by tumor promoters reflects their capacity to protect cells against at least one consequence of tumor promotion.

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LEGENDS FOR FIGURES

FIGURE 5.1 Effect of mannitol on ornithine decarboxylase activity

The scavenger was added directly to a confluent monolayer of C3H 10T1/2 cells grown in Dulbecco's modified medium supplemented with 10% (v/v) fetal bovine serum and 20mM HEPES one hour before gallate administration. After gallate administration the cells were incubated for another 5 hours, then the medium was removed. Cells were scraped, pelleted and stored at -70°C (for up to 15 days) until ornithine decarboxylase assay could be performed. "GA alone" (dark bars) stands for the effect of gallate; "+25mM MAN" (striped bars) refers to the condition of gallate plus 25mM mannitol; "+50mM MAN" (hatched bars) refers to the presence of 50mM mannitol with gallate. The controls ranged from 50-93 pmole CO_2 released per hour per mg protein between days. Error bars represent 8.1% of ornithine decarboxylase value for any concentration considered by repeatedly plotting the gallate-induced ornithine decarboxylase activity response curve (n>> 10 for each point considered) and determining the relative difference.

FIGURE 5.2 Effect of catalase on ornithine decarboxylase activity

The conditions were identical to those described for figure 1. "GA alone" (dark bars) stands for the effect of gallate acting alone; "+10U/ml CAT" (striped bars) and "+50U/ml CAT" (hatched bars) refer to 10U/ml and 50U/ml catalase (respectively) acting in combination with gallate. The controls ranged from 95-170 pmole CO_2 released per hour per mg protein between days.

FIGURE 5.3 Effect of superoxide dismutase on ornithine decarboxylase activity

The conditions were identical to those described for figure 1. "GA alone" (dark

bars) stands for the effect of gallate acting alone; "+10U/ml SOD" (striped bars) and "+50U/ml SOD" (hatched bars) refers to 10U/ml and 50U/ml superoxide dismutase (respectively) acting in combination with gallate. The controls ranged from 95-186 pmole CO_2 released per hour per mg rotein between days. **FIGURE 5.4 Effect of formate on ornithine decarboxylase activity**

The conditions were identical to those described for figure 1. "GA alone" (dark bars) refers to the effect of gallate acting alone; "+50mM FORM" (striped bars) and "+75mM FORM" (hatched bars) refers to the condition of gallate plus 50mM formate and 75mM formate (respectively). The controls ranged from 66-95 between days.

FIGURE 5.5 Effect of benzoate on ornithine decarboxylase activity

The conditions were identical to those described for figure 1. "GA alone" (dark bars) refers to the condition where gallate is acting alone; "+50mM BENZ" (striped bars) and "+75mM BENZ" (hatched bars) indicates the presence of 50mM and 75mM benzoate, respectively, with gallate. The controls ranged from 45-60 between days.

FIGURE 5.6 Effect of desferrioxamine on ornithine decarboxylase activity

The conditions were identical to those described for figure 1. "GA alone" (dark bars) refers to gallate acting alone; "+50uM DES" (striped bars) and "+100uM DES" (hatched bars) refers to the presence of 50μM and 100μM desferrioxamine, respectively, with gallate. The control ranged from 63-114 between days.

FIGURE 5.7 Effect of cycloheximide on gallate-induced ornithine decarboxylase activity

Cycloheximide at 5μg/ml, 10μg/ml or 20μg/ml (represented on the graph as +5ug/ml, +10ug/ml and +20ug/ml respectively) was added along with 89.5μM gallic

acid to the C3H 10T1/2 cells for a 5 hour incubation period. The dark bars, indicate that the cycloheximide was added to the cells one hour before the gallic acid was added (1 hr (pre)) while the striped bars indicate that the cycloheximide and gallic acid were added to the C3H 10T1/2 cells at the same time (no (pre)). The controls ranged from 36-55 between days.

TABLE 5.1 Comparison of the effects of scavengers of active oxygen on the gallate-induced increase in ornithine decarboxylase activity and the rate of autoxidaiton of gallate

This table summarizes the effects of scavengers of active oxygen on the autoxidation rate of gallate as well as their effects on the gallate-induced increase in ornithine decarboxylase. Data for the effects of the various scavengers on the autoxidation rate of gallic acid are replotted from the appendix. Superoxide dismutase and catalase were at a concentration of 250U/ml, benzoate was at a concentration of 50mM and desferrioxamine was at a concentration of 1mM, and gallic acid was present at a concentration of 2mM. The experimental conditions for the autoxidation studies were: pH 8.5, temperature 25°C, carried out in air-saturated 0.5M HEPES buffer. The scavenger concentration and experimental conditions for the ornithine decarboxylase activity experiments, are as indicated in figures 5.1-5.6.

FIGURE 5.1

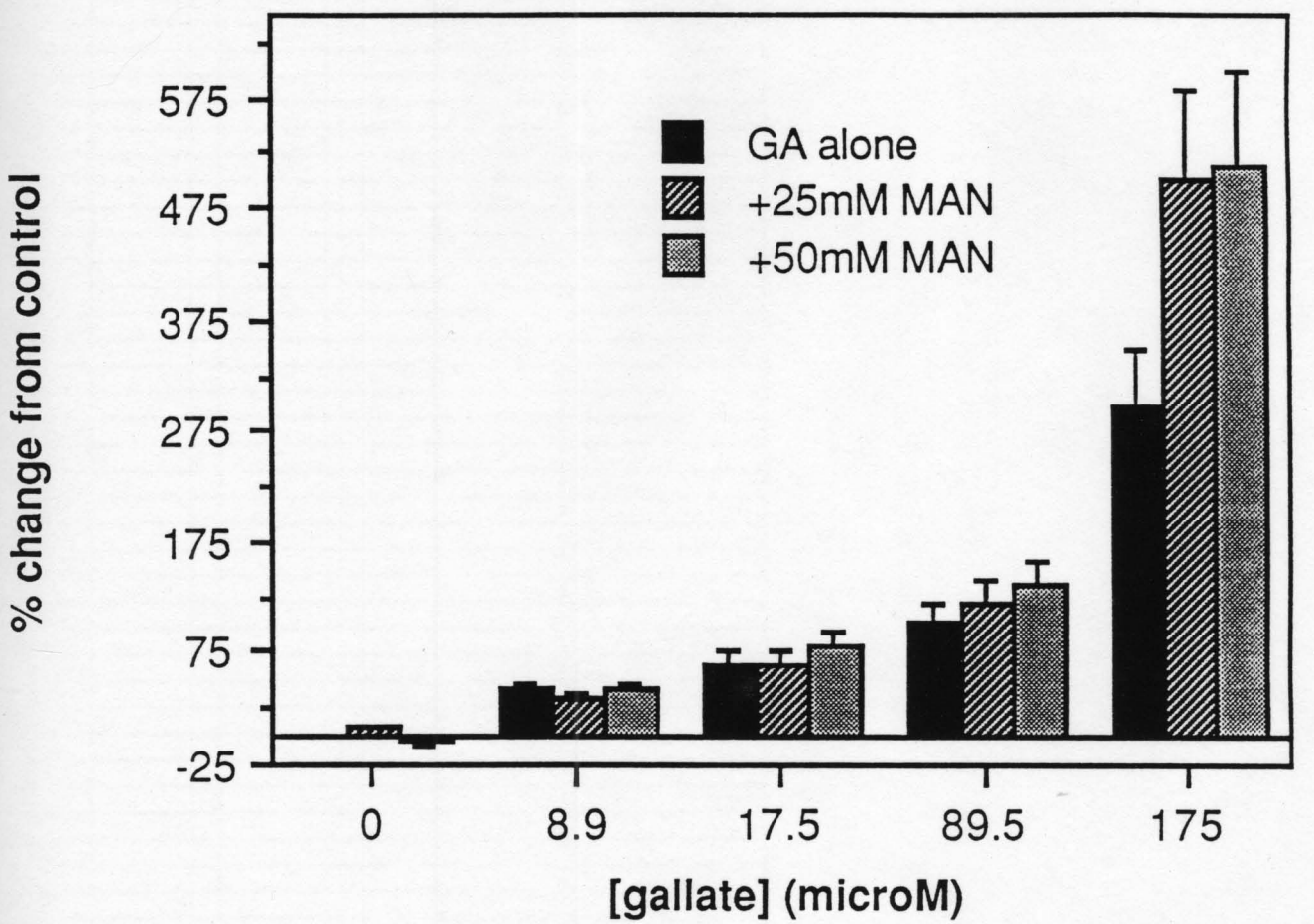
% change from control
575
475
375
275
175
75
-25
GA alone
+25mM MAN
+50mM MAN
0
8.9
17.5
89.5
175
[gallate] (microM)

FIGURE 5.2

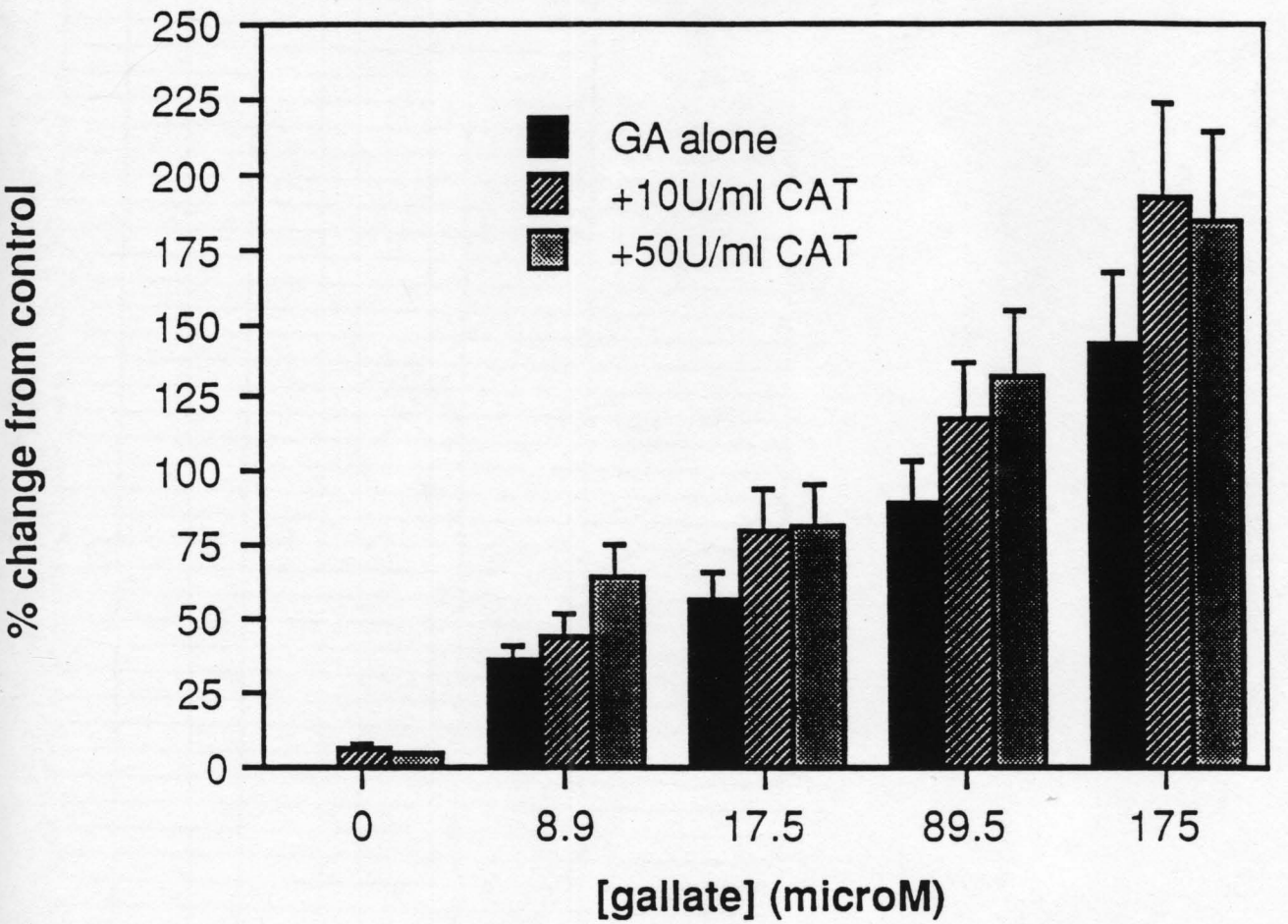
250
225
200
175
150
125
100
75
50
25
0
% change from control
GA alone
+10U/ml CAT
+50U/ml CAT
0
8.9
17.5
89.5
175
[gallate] (microM)

FIGURE 5.3

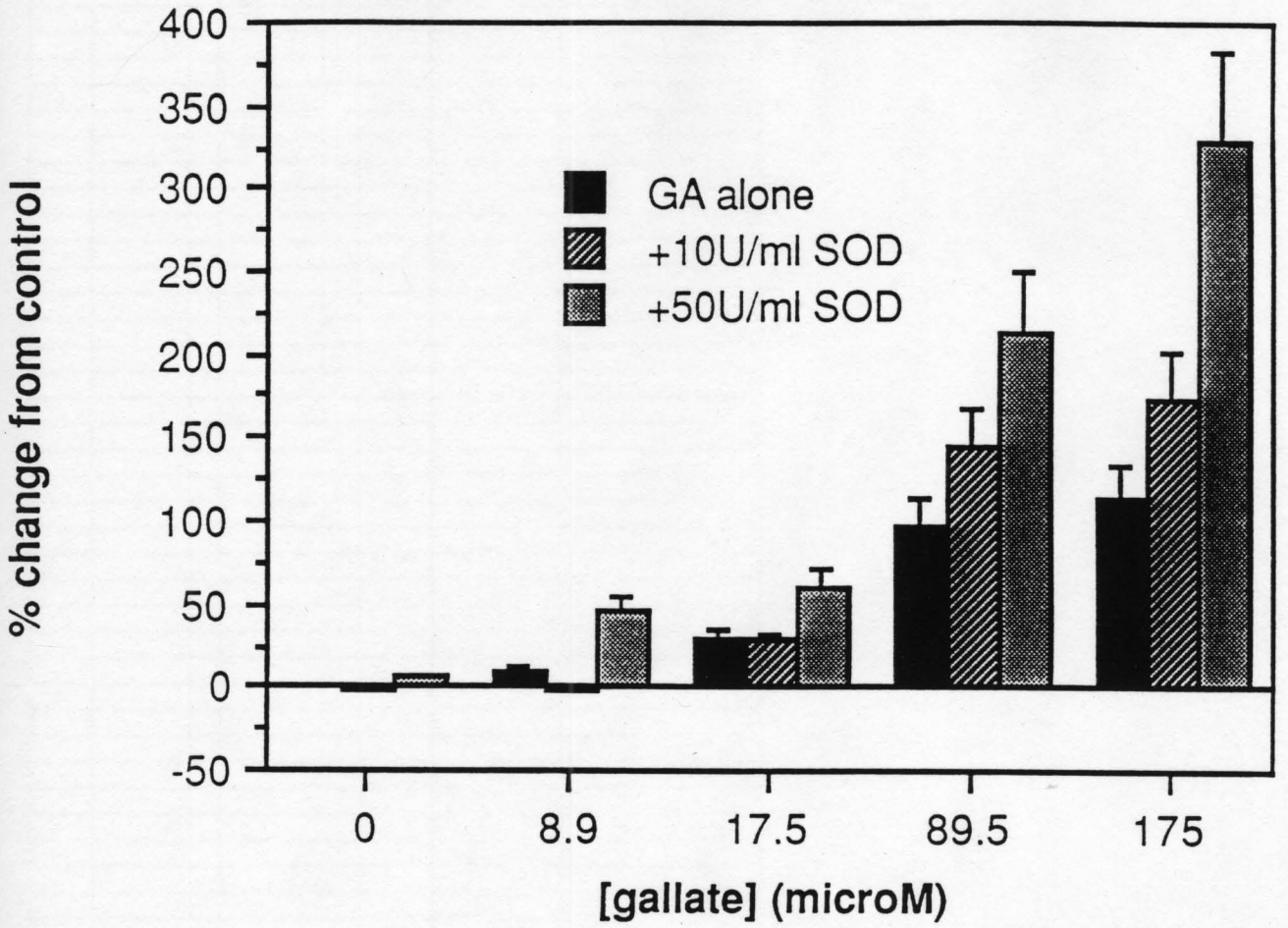
400
350
300
250
200
150
100
50
0
-50
% change from control
GA alone
+10U/ml SOD
+50U/ml SOD
0
8.9
17.5
89.5
175
[gallate] (microM)

FIGURE 5.4

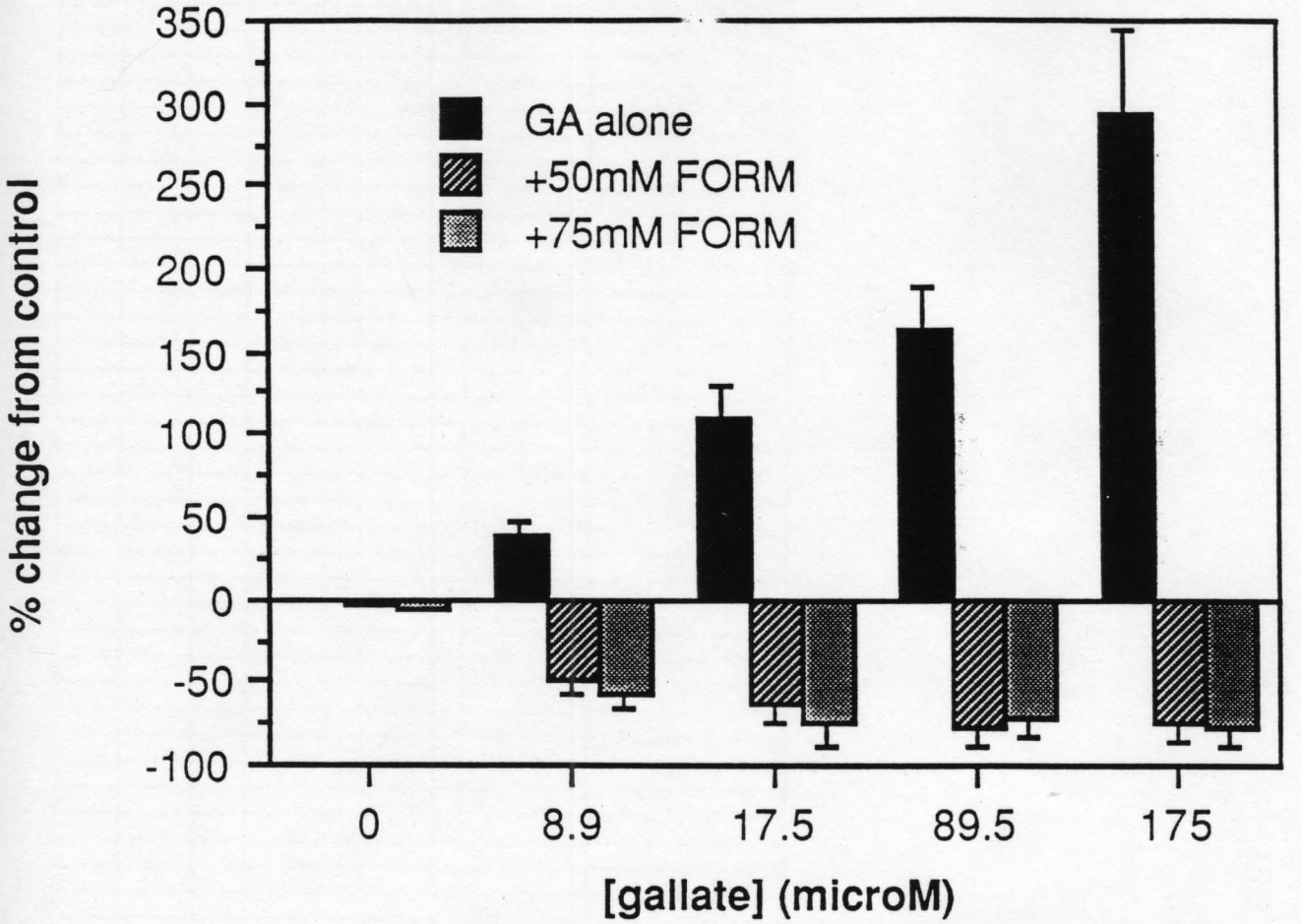
350
300
250
200
150
100
50
0
-50
-100
% change from control
GA alone
+50mM FORM
+75mM FORM
0
8.9
17.5
89.5
175
[gallate] (microM)

FIGURE 5.5

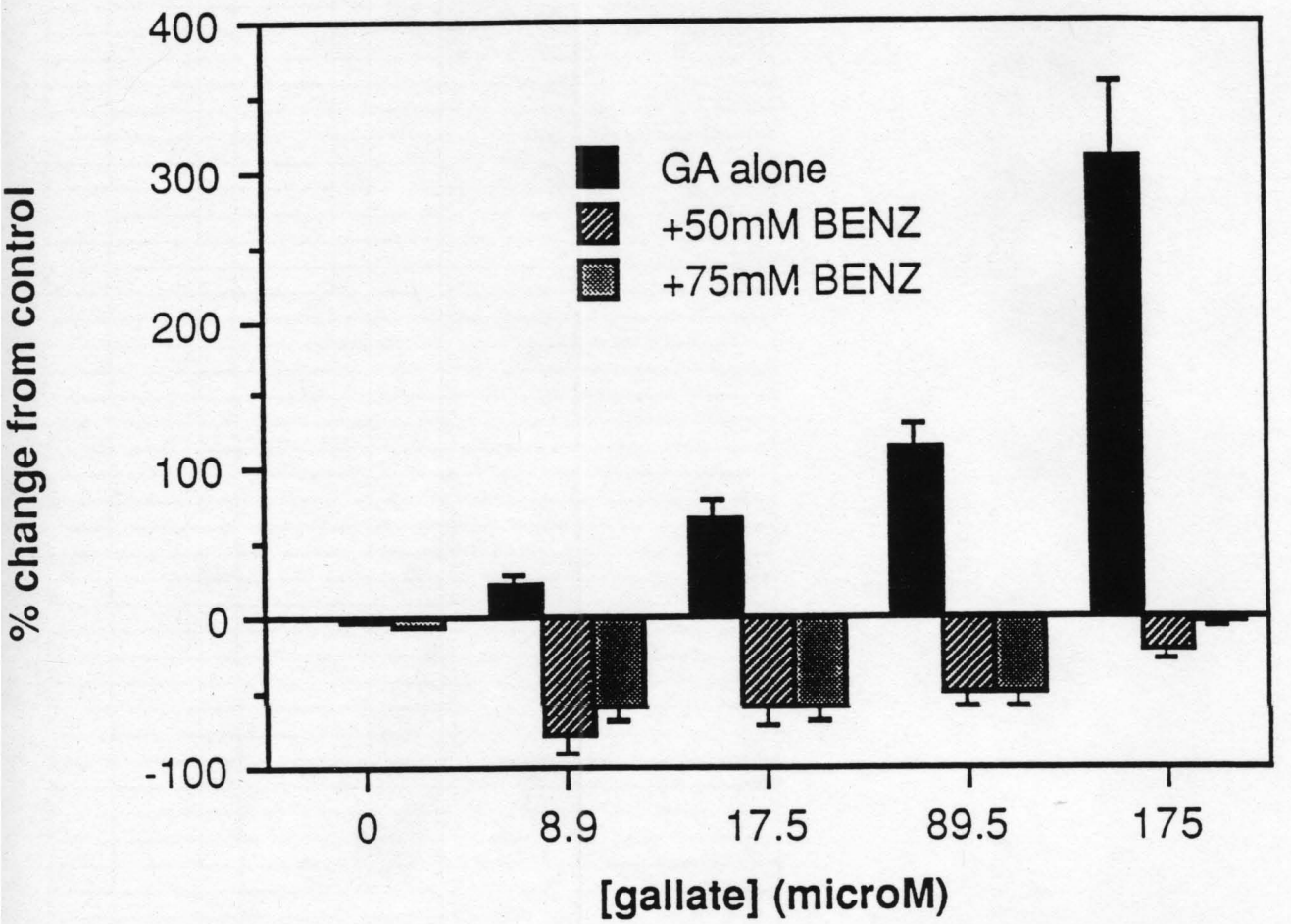
400
300
200
100
0
-100
% change from control
GA alone
+50mM BENZ
+75mM BENZ
0
8.9
17.5
89.5
175
[gallate] (microM)

FIGURE 5.6

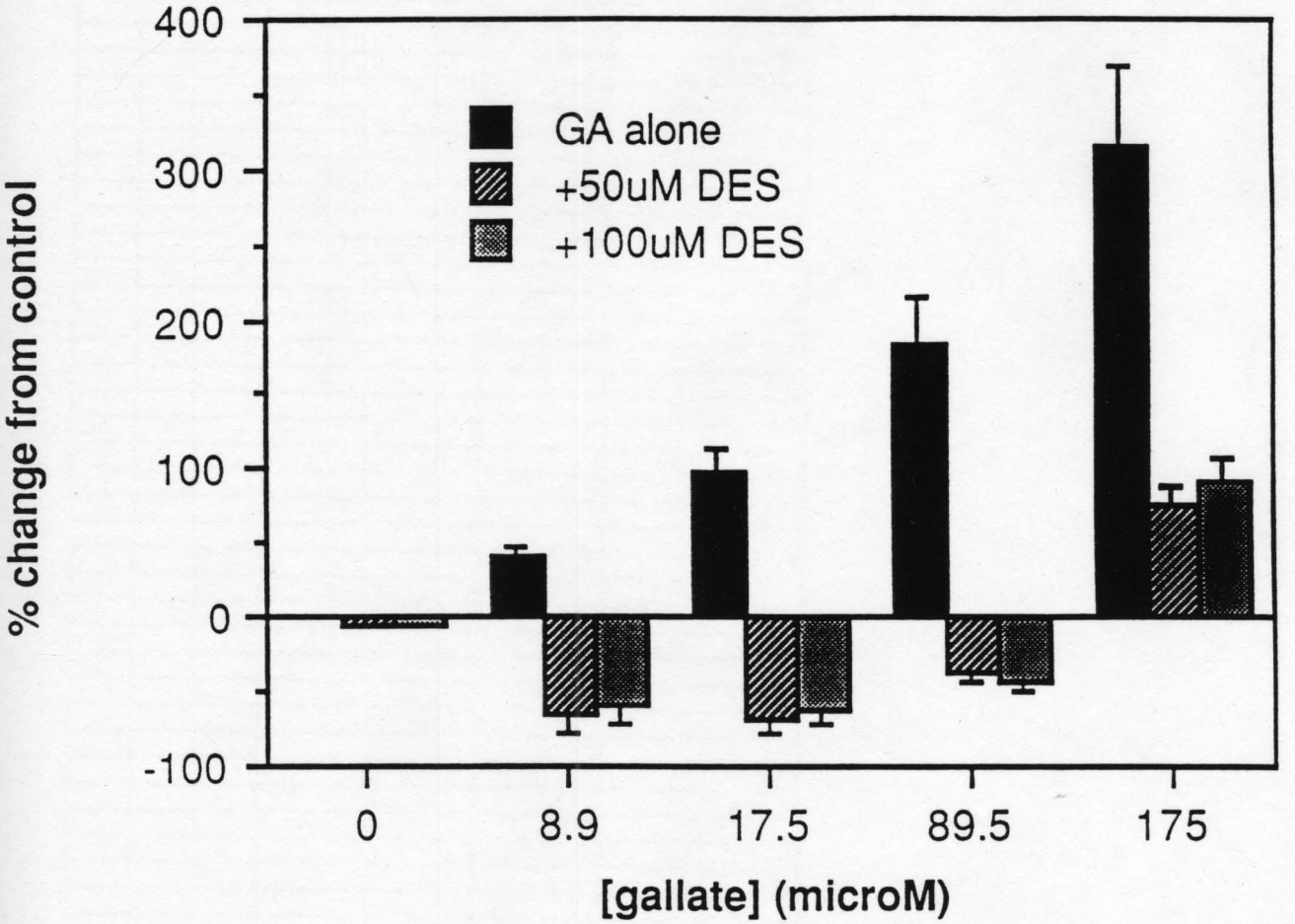
400
300
200
100
0
-100
% change from control
GA alone
+50uM DES
+100uM DES
0
8.9
17.5
89.5
175
[gallate] (microM)

FIGURE 5.7

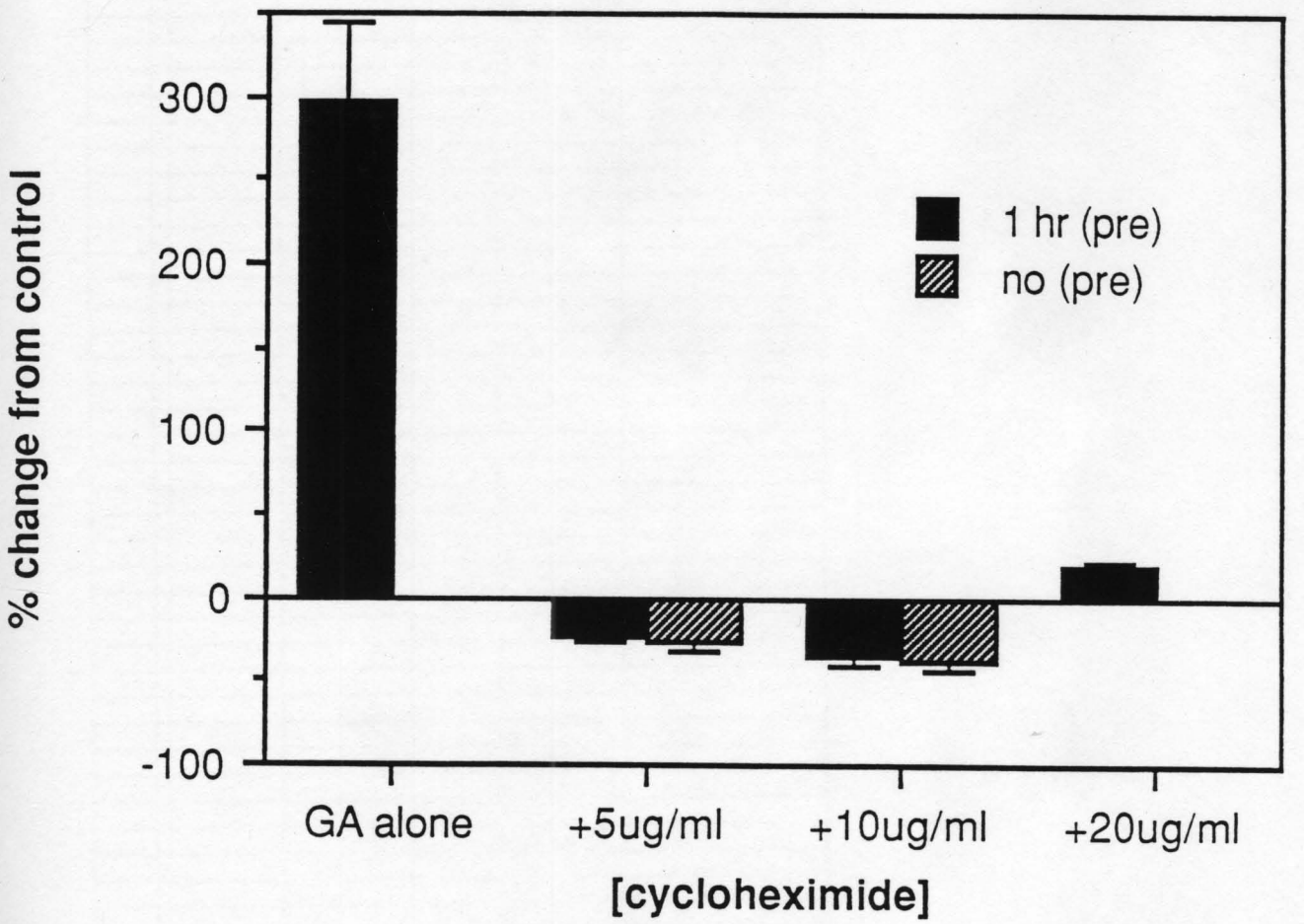
% change from control
300
200
100
0
-100
1 hr (pre)
no (pre)
GA alone
+5ug/ml
+10ug/ml
+20ug/ml
[cycloheximide]

Table 5.1

Scavenger used	Effect of scavenger on rate of autoxidation of gallate	Effect on gallate-induced ODC activity (current study)
Superoxide dismutase	inc. 12%	inc. 215% (50U/ml)
Catalase	inc. 9%	inc. 42% (50U/ml) no effect (10U/ml)
Mannitol	no data	inc. 200%
Formate	no data	decr. 370%
Benzoate	inc. 21%	decr. 330%
Desferrioxamine	decr. 61%	decr. 230%

CHAPTER VI

GENERAL CONCLUSIONS

There is a measure of correspondence between the induction of cellular ornithine decarboxylase activity seen in the current study, and the previously documented mutagenic and clastogenic actions of the phenolics. Further studies are necessary to correlate the effects of phenolics on mammalian systems with changes in ornithine decarboxylase activity, papilloma formation and clastogenic activity. Conflicting reports of genotoxicity of some phenolics *in vivo* may be resolved when more appropriate comparisons are made using a wide range of concentrations.

Comparing the effects of scavengers on rates of autoxidation of gallate *in vitro* we conclude that the stimulation of ornithine decarboxylase activity caused by gallate is closely tied to the autoxidation of gallate. The effects of hydroxyl radical scavengers suggest that the production of hydroxyl radicals may be largely responsible for the ability of gallate to stimulate ornithine decarboxylase activity.

Meanwhile, the response of ornithine decarboxylase to proxidants is a useful screening procedure for their potential tumor promoting effects. Moreover, the effects of scavengers and antioxidants on promoter stimulated ornithine decarboxylase activity may predict their effectiveness as chemoprotective agents against tumor promotion.

The response is inhibited by at least one agent which blocks *de novo* protein synthesis. This suggests that the induction of ornithine decarboxlase by gallate involves synthesis of new protein rather than any free radical mediated

activation of pre-existing enzyme. We do not know at this time whether this involves regulation at the transcriptional or translational levels. Future studies should focus on the more precise identification of the species involved in the regulation of ornithine decarboxylase activity, and elucidation of the precise step in synthetic control mechanisms at which the species act. Correlations with proxidant and antioxidant activity of phenolics are also needed. The ease and the relatively rapid turn around time of results indicates that the ornithine decarboxylase activity assay may be a good system in future to clarify these points.

APPENDIX

ROLES OF ACTIVE OXYGEN SPECIES IN THE AEROBIC OXIDATION OF GALLIC ACID, AND THE EFFECTS OF SCAVENGERS OF ACTIVATED OXYGEN SPECIES AND METAL CHELATORS

by

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Running title:

AEROBIC OXIDATION OF GALLIC ACID

Abbreviations used: EDTA, ethylenediaminetetracetate; BSA, bovine serum albumin.

ABSTRACT

Rates of oxidation of gallic acid (3,4,5-trihydroxybenzoic acid) were determined in the presence of mixtures of catalase (250 U/ml), superoxide dismutase (250 U/ml), benzoate (50 mM), desferrioxamine (1 mM), and ethylenediaminetetracetate (EDTA) (1 mM) by measuring the rate of appearance of quinonoid product at 630 nm. When added individually, superoxide dismutase and catalase, did not significantly accelerate oxidation, whereas the addition of benzoate significantly increased the oxidation rate by 21%. EDTA did not significantly decrease the oxidation rate whereas the addition of desferrioxamine induced a 61% decrease. Addition of scavengers and chelating agents in combination produced more complicated effects. Desferrioxamine or EDTA together with other scavengers invariably decreased the oxidation rate. In contrast to their effects when added alone, superoxide dismutase or catalase slowed the oxidation when desferrioxamine or EDTA were present. Benzoate accelerated oxidation when added to mixtures containing any combination of scavengers. The data support the view that transition metal ions play a major role in the reaction of gallic acid with molecular oxygen, to the point where propagation by active species of oxygen is detectible only if contaminant traces of metal ions are bound by chelating agents.

Keywords: gallic acid, oxidation, metal ions, free radicals, free radical scavengers, superoxide dismutase, catalase.

INTRODUCTION

Gallic acid (3,4,5-trihydroxybenzoic acid) is a phenolic compound found free in low concentrations in a wide variety of plant species. Vegetable tannins and other bound forms are thought to be the main sources of gallic acid in foods[1,2]. The antioxidant properties of gallic acid have been known since the 1940s[3] The propyl ester form in particular is widely used in the processing of foodstuffs susceptible to autoxidative damage[3,4]. Although numerous studies have supported the relative harmlessness of gallates[5,6,7,8], several recent workers have emphasized the fact that the metabolism of plant-derived phenolic compounds in animals may produce toxic effects under certain conditions [2,9].

There are conflicting reports concerning the biological effects of gallic acid. Clinically it has been used as an anti-inflammatory agent[10] and to protect against radiation damage[11]. As in the case of other phenolic compounds however, therapeutic benefits and toxicity frequently co-exist[2]. Gallic acid potentiates epinephrine toxicity by inhibiting catechol O-methyl transferase[12,13]. It inhibits hepatic lipogenesis and gluconeogenesis[14], antagonizes the effects of bradykinin on smooth muscle[15], suppresses certain aspects of the immune response[16], and may produce an allergic contact dermatitis[17]. In addition, although gallic acid has been reported to be non-mutagenic[18] and to be capable of decreasing the clastogenic effects of model nitrosation systems[19] it has been shown to be genotoxic in mammalian cell lines[20]. The acute toxicity of gallic acid is apparently lower than that of some other triphenols, since relatively large oral doses are required to induce toxicity in laboratory animals[7,8].

The actions of plant-derived phenolic compounds are complex, in that neither pro-nor antioxidant properties can account for all of their observed effects[1,21]. Interactions between quinonoid reaction products, oxygen species and metal ions are crucial in determining whether the net effects are protective or damaging. Gallic acid is oxidized by molecular oxygen, particularly in the presence of enzymes such as polyphenol oxidase[22]. Oxidation of gallic acid results in the formation of quinonoid products[23] while the concurrent reduction of molecular oxygen leads to a variety of radical species. Both quinonoid products and activates oxygen may then participate in further free radical chain reactions. For example, further reactions of gallic acid with the product (H_2O_2) involve reactive species including singlet state oxygen, as revealed by chemiluminescence[24].

In the hope of gaining insight into the mechanisms underlying free radical generation in the gallic acid/O_2 reaction and the role(s) of transition metals, we investigated the effects of selected free radical scavengers, (individually and in combination), and of metal chelating agents on the rate of reaction of gallic acid with molecular oxygen.

MATERIALS AND METHODS

Reagents

All experiments were performed at 25°C using air-saturated 0.5 M HEPES buffer (N-2-hydroxyethylpiperazine-N'-2-ethansulfonic acid) (Sigma Chemical Co., St. Louis, Mo) prepared with double distilled water and titrated to pH 8.5 using KOH. Other reagents purchased from Sigma Chem. Co. included gallic acid, catalase EC 1.11.1.6 (bovine liver, 1860 U per mg), superoxide dismutase EC 1.15.1.1 (2800 U per mg) and bovine serum albumin. Sodium benzoate was purchased from Anachem Chemical Ltd. (Toronto, Canada), and ethylenediamintetracetate (EDTA) from Fisher Scientific Co., Fairlawn N.J. Desferrioxamine (Desferyl mesylate) was a gift from CIBA Pharmaceutical Co., Summit N.J.

Preparation of Anaerobic Solutions of Gallic Acid

Nitrogen was bubbled through 10.0 ml of 0.5M pH 8.5, HEPES buffer contained in a rubber capped vial for a period of 10 minutes prior to the addition of gallic acid to ensure nitrogen saturation of the buffer. A measured quantity of dry gallic acid was introduced into the buffer solution as quickly as possible followed by another nitrogen purge. The vial was then sealed under a slight positive pressure of nitrogen. The vial was subsequently wrapped in aluminum foil, in order to minimize the destructive effects of ambient light, and magnetically stirred for 45 minutes to ensure complete solution of gallic acid.[1]

Experimental Procedure

Air-saturated HEPES buffer (0.5M pH 8.5) maintained at 25°C, together with various combinations of scavenger substances were added to the sample cuvette to a final volume of 2.45 ml. The reaction was then initiated by the addition of 50μl of gallic acid stock solution, to a final concentration of 2mM. The rate of the reaction determined by recording the change in absorbance at 630 nm. The selection of this particular wavelength was made on the basis of results from pilot studies in which both the UV and visible regions of the spectrum were scanned in order to determine the change in spectra during the autoxidation of gallic acid. The results obtained were in agreement with those of Fink et. al., 1982.[25]

Data Analyses and Statistics

Data analyses were performed using programs written locally in APL and implemented on an IBM 3081 mainframe computer to calculate initial rates by linear regression of the initial (first order) portions of the reaction profile curves. Because the variability between duplicates was roughly proportional to the reaction rate, statistical analysis was based on the relative-standard error derived from the control reaction. This value was then used to estimate the standard error for all other conditions.

RESULTS

Effects of Individual Scavengers

The effects of individual scavengers on the rates of autoxidation of gallic acid are shown in Figure 1. Unexpectedly, catalase and superoxide dismutase did not significantly change the oxidation rate, whereas benzoate increased the rate of oxidation by 21%. In contrast, metal chelating agents decreased the oxidation rate, desferrioxamine decreasing the rate by 61% and EDTA decreasing it by 12%. Also evident in Figure 1 is the variability of the effects of a given scavenger depending on the other test reagents present. The remainder of this section will discuss the effects of other test reagents, simultaneously present, on the actions of each of the scavengers or metal chelating agents.

Effects of the Presence of Other Scavengers on the Action of Superoxide Dismutase

It is apparent from Figure 2 that superoxide dismutase may either increase or decrease the rate of gallic acid oxidation, depending on which other scavengers and/or chelating agents are present. If catalase or benzoate was present, superoxide dismutase increased the rate of oxidation by 29% or 33%, respectively, whereas its addition in the presence of a mixture of catalase plus benzoate did not significantly change the oxidation rate over that seen in the system containing just catalase plus benzoate (superoxide dismutase absent). In contrast, superoxide dismutase inhibited gallic acid oxidation when desferrioxamine was present in the mixture on its own, together with benzoate, or with catalase by 17%, 19%, or 14% respectively. Addition of superoxide dismutase to a solution containing EDTA led to a 13% decrease in oxidation rate, whereas addition of

superoxide dismutase to a system containing EDTA plus catalase or EDTA plus benzoate did not significantly alter the oxidation rate of the particular system in the absence of superoxide dismutase.

Effects of the Presence of Other Scavengers on the Actions of Catalase

The results presented in Figure 3 indicate that catalase, like superoxide dismutase, caused either an increase or decrease in the rate of oxidation of gallic acid. These contrasting effects depended, once again, on the presence of desferrioxamine or EDTA. Addition of catalase to reaction mixtures containing superoxide dismutase or benzoate accelerated the autoxidation by 26% or 20%, respectively, whereas its addition to a system containing both superoxide dismutase and benzoate did not significantly change the reaction rate. Addition of catalase to mixtures containing benzoate and desferrioxamine decreased the oxidation rate by 8%, but the addition of catalase to a mixture containing EDTA (or desferrioxamine or superoxide dismutase) plus desferrioxamine did not significantly alter the oxidation rate. In contrast, addition of catalase to benzoate plus EDTA or superoxide dismutase plus EDTA produced 22% and 13% increases, respectively, in the oxidation rate.

Effects of the Presence of Other Scavengers on the Actions of Benzoate

Benzoate on its own increased the rate of oxidation of gallic acid. Its action was unique in the sense that it increased the oxidation rate regardless of which other scavengers or chelators were present. The only exception, seen in Figure 4, was its effect in the mixture containing EDTA alone where there was no change in the oxidation rate. The greatest increases, 42% and 32%, were seen when benzoate was added to reaction mixtures containing either superoxide

dismutase or catalase. In reaction mixtures containing EDTA in combination with superoxide dismutase or catalase, addition of benzoate produced increases of 26% and 28%, respectively. Benzoate added to solutions containing desferrioxamine, catalase plus desferrioxamine or superoxide dismutase plus desferrioxamine produced small but statistically significant increases in oxidation rate of 9%, 7% and 7%, respectively.

Effects of the Presence of Other Scavengers on the Actions of EDTA and Desferrioxamine

A comparison of Figure 5 with Figure 6 shows that addition of either EDTA or desferrioxamine decreased the rate of oxidation of gallic acid when in combination with any of the active oxygen scavengers (benzoate, catalase, superoxide dismutase, or combinations of these). The effects of EDTA were less striking than those of desferrioxamine. EDTA added to reaction mixtures containing desferrioxamine, catalase, benzoate plus catalase, benzoate, superoxide dismutase, catalase plus superoxide dismutase or benzoate plus superoxide dismutase decreased the oxidation rates by 14%, 21%, 31%, 33%, 38%, 51%, and 54.2%, respectively. Greater decreases were seen upon the addition of desferrioxamine to the systems containing EDTA (63%), benzoate (73%), catalase (76%), superoxide dismutase (90%), benzoate plus catalase (101%), catalase plus superoxide dismutase (118%) and benzoate plus superoxide dismutase (125%). The change in oxidation rate seen upon addition of both chelating agents together did not appreciably exceed the sums of their individual contributions, (figures 5 and 6 seventh bar from the left and figure 1).

Identification of the Roles of Selected Reaction Intermediates

The roles of specific active oxygen species, specifically superoxide, hydrogen peroxide and hydroxyl radicals, in the oxidation of gallic acid were studied in three selected systems containing (1) desferrioxamine plus catalase plus benzoate, (2) desferrioxamine plus benzoate plus superoxide dismutase, and (3) desferrioxamine plus catalase plus superoxide dismutase, respectively. As shown in Figure 7, addition of each of these mixtures caused a decrease in the oxidation rate (60%, 71% and 80%). Comparing these results with the inhibition by desferrioxamine alone (61%) excludes the possibility that activated oxygen plays a major catalytic role, whether as chain reaction initiators or propagators. (When EDTA replaced desferrioxamine as the metal chelator the addition of the above three mixtures did not significantly alter the rate of oxidation from that of gallic acid alone). This conclusion was confirmed by the effects of a mixture containing superoxide dismutase plus catalase plus benzoate. The 59% increase in the rate of oxidation of gallic acid resulting from removal of most plausible forms of chain propagating species of oxygen confirm a major dependence in the oxidation of gallic acid, on transition metal ions as chain initiating or propagating species.

DISCUSSION

Like other autoxidations involving phenolic compounds, the autoxidation of gallic acid is complicated. The extent of participation of a potential reaction intermediate may be reflected in the extent to which the rate of autoxidation observed in mixtures containing scavengers for all likely active species except this agent differ from the rate in the presence of the most inhibitory combination of scavengers. This difference reflects an estimate of the contribution of the selected species.

Effects of Metal Chelating Agents

In all cases, desferrioxamine decreased the rate of oxidation by greater than 50%, reflecting a substantial contribution of transition metal ions (particularly iron) in the reaction of gallic acid with molecular oxygen. That EDTA caused smaller decreases in the rate of oxidation reflects a smaller contribution of most transition metal ions other than iron. The differing effects of these chelators on the actions of a specific scavenger of active oxygen reflect their distinct influences on the environment and redox state of metal ions in the system.

Effects of Superoxide Dismutase

The increase in rate of oxidation of gallic acid caused by superoxide dismutase could be due either to the H_2O_2 produced via the dismutation reaction, or to the elimination of some inhibitory action of superoxide, such as removal of an important reaction intermediate. Conversely, when superoxide dismutase was added to mixtures that contained one or both of the metal chelating agents (except for EDTA plus benzoate or EDTA plus catalase), the oxidation rate was decreased. This contrasts with other O_2^- dependent autoxidations, where the

presence of EDTA confers increased sensitivity to inhibition by superoxide dismutase.[26,27] Figure 2 confirms that the addition of superoxide dismutase to desferrioxamine and to EDTA containing solutions is again inhibitory. These results may indicate then, that although metals play a major role in propagating the oxidation of gallic acid, O_2^- plays a subordinate role which becomes detectable when metal ions are not available.

Effects of Catalase

Addition of catalase to most test mixtures (exceptions were those containing desferrioxamine and those containing only EDTA, where catalase decreased the rate of oxidation) produced an increase in rate of at least 10%. This effect may be due to the relatively slower decrease in the concentration of molecular oxygen as a result of regeneration of O_2 from H_2O_2 in the presence of catalase. The decreases in rate caused by catalase in the presence of desferrioxamine and EDTA may reflect the loss of the contribution of Fenton-type reaction mechanisms.

Effects of Scavengers of Hydroxyl Radicals

The roles of hydroxyl radicals in this system remain unclear, since the addition of benzoate, a hydroxyl radical scavenger, caused an increase in the rate of oxidation in almost every reaction in which it was included (the only exception was in the presence of EDTA alone). Since hydroxyl radicals are unlikely to hinder oxidation, we conclude that the products of hydrogen atom abstraction from benzoate may stimulate oxidation. More work is needed to establish the precise roles of hydroxyl radicals and their scavengers.

Comparison With Autoxidation of Other Triphenols

The current results contrast sharply with the similar studies performed on the autoxidation of other di and triphenols [28,29,30,31,32]. In the case of 6-OHDA, superoxide plays a primary role in propagating the reaction with oxygen, whereas metal ions play a secondary role, and other reduced oxygen species (such as H_2O_2 or hydroxyl radicals) play minor stimulatory roles. The superoxide anion has also been identified as a major propagating species, in the autoxidation of epinephrine and pyrogallol[26,27]. In all three systems, 6-OHDA, epinephrine and pyrogallol metal ions act via alternate pathways whose importance is greater at lower, lower, and higher pH values, respectively [26,27]. In contrast, in the case of gallic acid, redox-active transition metal ions fulfill both the primary initiating and propagating roles.

These differences are accentuated by the differing effects of the addition of EDTA plus SOD upon the oxidation of epinephrine, pyrogallol and gallic acid. The EDTA-metal ion complex formed reacts with oxygen by an outer sphere mechanism to generate superoxide, which accounts for EDTA's ability to sensitize the oxidation of epinephrine and pyrogallol to SOD. Its failure to do this in the gallic acid system reinforces the ideas stated above. Any contributions of reduced oxygen species, including superoxide, hydroxyl radicals and H_2O_2, to the overall rate of reaction of gallic acid with oxygen are kinetically inaccessible, occurring subsequent to the rate determining step.

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LEGEND FOR FIGURES

Fig. 1 **The effects of various combinations of scavengers of active oxygen and metal chelating agents on the oxidation rate of gallic acid.** The reaction cuvettes contained 0.5M HEPES buffer, pH 8.5, and various combinations of the scavengers of active oxygen and metal chelating agents; C: catalase, 250 U/ml; S: superoxide dismutase, 250 U/ml; B: benzoate, 50mM; D: desferrioxamine, 1mM; and E: ethylenediaminetetracetic acid, 1mM. The reactions were initiated by the addition of GA: gallic acid, final concentration 2mM, in a final volume of 2.5 ml. All reactions were performed at 25°C. The percent change represents the percent difference in the oxidation rate of the reaction mixture under study when compared to the oxidation rate of gallic acid alone. Error bars represent the standard errors as calculated from the relative-standard error for the control reaction.

Fig. 2 **The effects of superoxide dismutase on the oxidation rate of gallic acid.** The experimental conditions were as outlined in fig. 1. The results presented are the percent changes in the oxidation rate of a given mixture, observed upon the addition of superoxide dismutase. "NONE" refers to the reaction condition where gallic acid is the only compound present before the addition of superoxide dismutase.

Fig. 3 **The effects of catalase on the oxidation rate of gallic acid.** The experimental conditions and abbreviations are as indicated in fig. 1. The results presented are the percent changes in the oxidation rate of a given mixture, observed upon the addition of catalase. "NONE" refers to the reaction condition where gallic acid is the only compound present before the addition of catalase.

Fig. 4 **The effects of benzoate on the oxidation rate of gallic acid.** Experimental conditions and abbreviations as indicated in fig. 1. The results presented are the percent changes in the oxidation rate of a given mixture, observed upon the addition of benzoate. "NONE" refers to the reaction condition where gallic acid is the only compound present before the addition of benzoate.

Fig. 5 **The effects of EDTA on the oxidation rate of gallic acid.** Experimental conditions and abbreviations as stated in fig. 1. The results presented are the percent changes in the oxidation rate of a given mixture, observed upon the addition of EDTA. "NONE" refers to the reaction condition where gallic acid is the only compound present before the addition of EDTA.

Fig. 6 **The effects of desferrioxamine on the oxidation rate of gallic acid.** Experimental conditions and abbreviations as indicated in fig. 1. The results presented are the percent changes in the oxidation rate of a given mixture, observed upon addition of desferrioxamine. "NONE" refers to the reaction condition where gallic acid is the only compound present before the addition of desferrioxamine.

Fig. 7 **The effects of superoxide, metals, hydrogen peroxide and the hydroxyl radical on the rate of oxidation of gallic acid.** The experimental conditions and calculations are as described in fig. 1. The role of superoxide, (role of O2-), was estimated from the reaction rate in the presence of a mixture containing: benzoate plus catalase plus either desferrioxamine or EDTA as indicated in the legend on the graph. The role of metals, (role of M+), was estimated from the reaction rate in the presence of: benzoate plus catalase plus superoxide dismutase, while the role of hydrogen peroxide, (role of H2O2), was estimated from the

reaction rate in the presence of a mixture of: benzoate plus superoxide dismutase and one of the metal chelators, desferrioxamine or EDTA, as indicated on the graph. The role of the hydroxyl radical, (role of $OH^{\bullet}$), was estimated from the reaction rate in the presence of catalase plus superoxide dismutase plus either desferrioxamine or EDTA, as indicated in the legend.

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