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City University of New York PH.D. 1985

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IN VITRO DEVELOPMENT OF 2,4-DOPA, A TYROSINASE-ACTIVATED PRODRUG OF POTENTIAL USE IN THE TREATMENT OF MALIGNANT MELANOMA

by

MARK EDWIN MORRISON

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

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Abstract

IN VITRO DEVELOPMENT OF 2,4-DOPA, A TYROSINASE-ACTIVATED PRODRUG OF POTENTIAL USE IN THE TREATMENT OF MALIGNANT MELANOMA

by

Mark Edwin Morrison

Adviser: Dr. Gerald Cohen

Tyrosinase (monophenol, dihydroxyphenylalanine:oxygen oxidoreductase, E.C. 1.14.18.1) is found in the melanosomes of normal and malignant pigment cells and catalyzes the hydroxylation of L-p-tyrosine in the meta-position to form L-3,4-DOPA and, ultimately, melanin pigment. In the present studies, tyrosinase was demonstrated to hydroxylate 2,4-DOPA (2,4-dihydroxyphenylalanine) in the meta-position to generate 6-hydroxy-DOPA (2,4,5-trihydroxyphenylalanine). 2,4-Dopamine was also tested as a substrate for tyrosinase and was hydroxylated in the meta-position to generate 6-hydroxydopamine (2,4,5-trihydroxyphenylethylamine) as product. Both reactions were accelerated by the inclusion of L-3,4-DOPA which is the natural cosubstrate for tyrosinase. The products, 6-hydroxy-DOPA and 6-hydroxydopamine, are well-known neurotoxins that are taken up into catecholamine neurons and express neurotoxicity primarily through the production of toxic oxy-radicals.

2,4-DOPA was tested in cell culture to assess its potential as a tyrosinase-targeted prodrug against melanoma. Treatment with 2,4-DOPA produced cytotoxicity against both B-16 and Cloudman melanoma cultures. In experiments with B-16 melanoma cultures, 2,4-DOPA inhibited the synthesis of DNA, RNA, and protein in a dose- and time-

dependent manner.

MJY-Alpha mammary tumor and L-1210 leukemia were studied to examine non-tyrosinase-mediated effects. No toxicity was observed against either MJY-alpha mammary tumor or L-1210 leukemia cultures.

C-1300 neuroblastoma cells do not contain tyrosinase but do contain tyrosine hydroxylase, the enzyme which catalyzes the hydroxylation of L-p-tyrosine to L-3,4-DOPA within catecholamine neurons. Neuroblastoma cultures were tested to examine the possibility that tyrosine hydroxylase might generate 6-hydroxy-DOPA from 2,4-DOPA, with resultant cytotoxicity. 2,4-DOPA was shown to be non-specifically cytotoxic to neuroblastoma cultures. The cytotoxicity of 2,4-DOPA could not be blocked by either alpha-methyl-p-tyrosine or 3-iodo-tyrosine, two potent inhibitors of tyrosine hydroxylase. Since the cytotoxicity against the neuroblastoma cells appeared to be non-specific, it is hoped that catecholamine neurons might not be at risk during treatment with 2,4-DOPA.

These studies indicate that 2,4-DOPA functions as a tyrosinase-activated prodrug which may potentially be of use in the treatment of malignant melanoma.

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LIST OF ABBREVIATIONS

ATP ..... Adenosine triphosphate

BSA ..... Bovine serum albumin

cAMP ..... Adenosine 3',5'-cyclic monophosphate

cm ..... Centimeter

3,4-DHBA 3,4-Dihydroxybenzylamine

2,4-DOPA 2,4-Dihydroxyphenylalanine

2,4-Dopamine .. 2,4-Dihydroxyphenylethylamine

DTPA ..... Diethylenetriaminepentaacetic acid

EDTA ..... Ethlyenediaminetetraacetic acid

g ..... Gram

G2 ..... Second growth phase of the cell cycle

GGHB Gamma-glutaminyl-4-hydroxybenzene

HPLC ..... High performance liquid chromatography

$K_{activator}$ .... Apparent Michaelis constant for the cofactor-mediated activation of tyrosinase-catalyzed hydroxylations of substrate

K_m ..... Michaelis constant

MEM ..... Minimal Essential Medium

mg ..... Milligram

mL ..... Milliliter

mM ..... Millimolar

MSH ..... Melanocyte stimulating hormone

PBS ..... Phosphate-buffered saline

PCA ..... Perchloric acid

PTU ..... Phenylthiourea

PU ..... Phenylurea

TCA ..... Trichloroacetic acid

TU ..... Thiourea

uL ..... Microliter

uM ..... Micromolar

um ..... Micrometer

Unit ..... One unit of tyrosinase activity was defined as that amount of enzyme preparation which generated 1 umole 6-hydroxy-DOPA per minute from 1 mM 2,4-DOPA as substrate and 50 uM L-3,4-DOPA as cosubstrate in the presence of ascorbate (1 or 0.1 mM) at 22°C. in 50 mM phosphate buffer, pH 6.8, containing 100 uM DTPA (and also 0.1% sodium cholate when mammalian tyrosinase was studied).

V_{max} ..... Maximal velocity per unit of enzyme activity

Chapter 1: INTRODUCTION AND BACKGROUND

1.1: INTRODUCTION TO PRESENT STUDIES

Malignant melanoma cells possess an obvious characteristic which sets them apart from most cells. In 97.7% of all cases reported, the tumor is pigmented (Ariel 1981). Pigmentation is the result of L-p-tyrosine conversion to melanin by the enzyme tyrosinase (Figure 1-1). The presence of tyrosinase provides a means to target agents to melanoma cells. This concept has led to investigations examining the potential antitumor efficacies of several different prodrugs (see below).

The tyrosinase-activated prodrugs which have been previously tested appear to function predominantly or solely through the production of toxic quinones which act to inhibit sulfhydryl-dependent enzymes. In the present experiments, an alternative modality was tested, namely that tyrosinase might hydroxylate 2,4-DOPA or 2,4-dopamine to generate the potent oxy-radical generators, 6-hydroxy-DOPA or 6-hydroxydopamine, respectively (Figure 1-2). The potential of one of these compounds (2,4-DOPA) as a possible chemotherapeutic agent for the treatment of melanoma was also evaluated in cell culture.

1.2: MALIGNANT MELANOMA

Melanoma is a neoplasm of melanocytes and occurs predominantly upon sun-exposed areas of the skin. It has been documented by Hippocrates as early as the the fifth century B.C. (Roses et al. 1983). Melanoma is a major health concern, and the incidence rate of

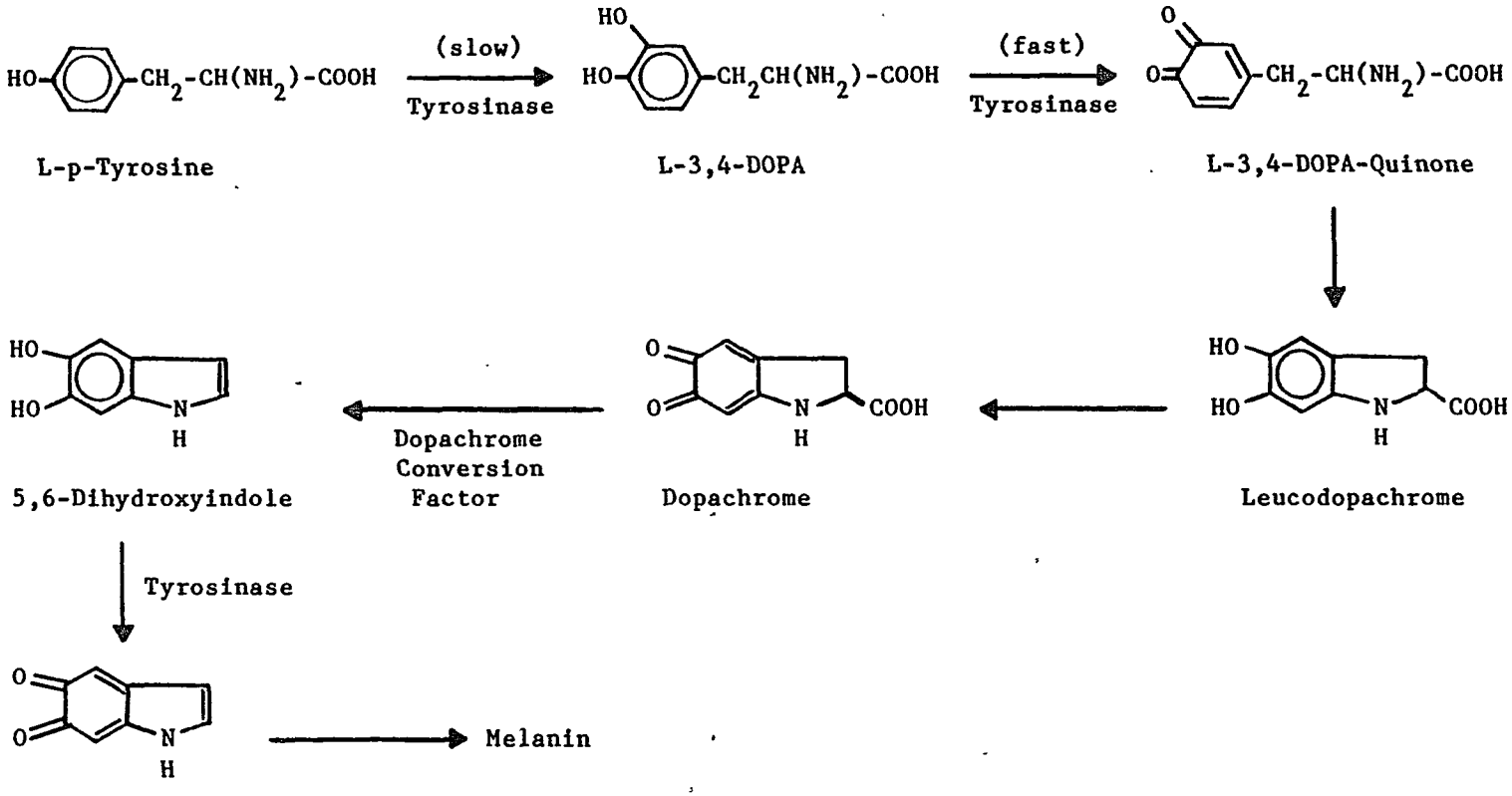


Figure 1-1. Synthesis of Melanin

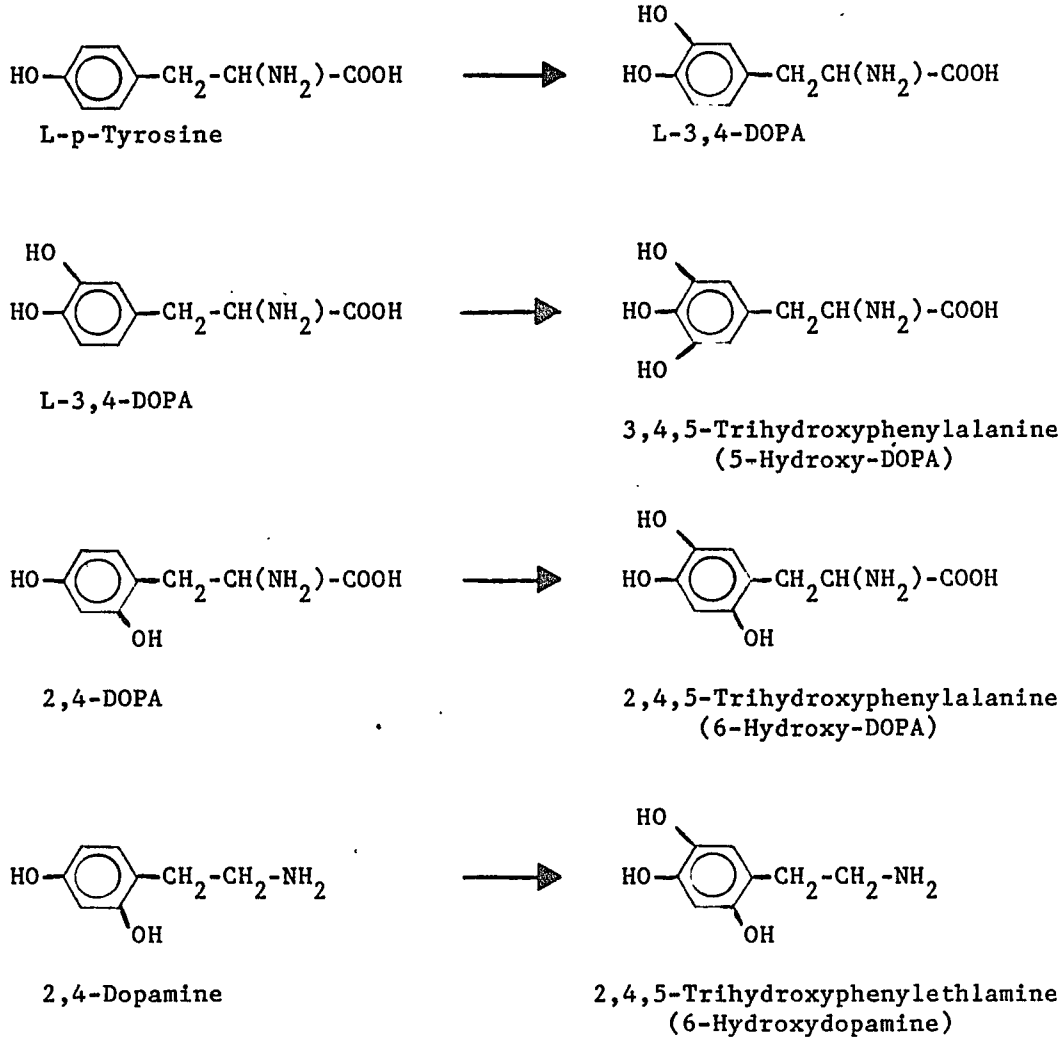


Figure 1-2. Tyrosinase-Catalyzed Hydroxylations

L-p-Tyrosine is the natural substrate for tyrosinase and is hydroxylated in the position meta to the side chain to generate L-3,4-DOPA which is subsequently oxidized to melanin pigment. A minor route in the production of melanin also involves the hydroxylation of L-3,4-DOPA in the alternate meta-position to generate 3,4,5-trihydroxyphenylalanine (5-hydroxy-DOPA).

The prodrugs, 2,4-DOPA and 2,4-dopamine, are also hydroxylated by tyrosinase meta to the side chain with the resultant generation of 2,4,5-trihydroxyphenylalanine (6-hydroxy-DOPA) and 2,4,5-trihydroxyphenylethylamine (6-hydroxydopamine), respectively.

melanoma has doubled in the past 10 years (Fitzpatrick et al. 1983). It has been estimated that in the United States alone, 17,700 new cases of melanoma will appear and 5,500 deaths will occur in 1984 (Silverberg 1984).

1.3: EXPERIMENTAL TYROSINASE-ACTIVATED PRODRUGS FOR THE TREATMENT OF MALIGNANT MELANOMA

1.3.1: Natural Tyrosinase Substrates as Targeted Prodrugs

Tyrosine

Interest in tyrosinase-activated prodrugs for the treatment of malignant melanoma began in 1963 when Hochstein and Cohen presented evidence that the rate of anaerobic glycolysis in Cloudman melanoma cells could be decreased by prior incubation in an aerobic medium containing tyrosine. However, if glucose was present, the rate of anaerobic glycolysis was almost unaffected by the addition of tyrosine. Tyrosine is converted to L-3,4-DOPA by tyrosinase within melanoma cells. L-3,4-DOPA, but not tyrosine, also inhibited glycolysis in brain homogenates. The mechanism by which L-3,4-DOPA inhibited glycolysis in brain homogenates was hypothesized to be mediated through the production of H_2O_2, since catalase was found to block the inhibition. The protection afforded by glucose in the experiments with melanoma cells was attributed to the operation of a glutathione peroxidase system, which was shown to metabolize H_2O_2 with the concomitant oxidation of glutathione. Glucose sustains the

activity of the hexose monophosphate shunt which generates NADPH. The NADPH, in turn, acts as an electron source for the reduction of oxidized glutathione by glutathione reductase, which was also shown by the authors to be present in melanoma cells. The authors concluded that melanization results in the generation of H_2O_2, which can be removed via the action of glutathione peroxidase.

An ancillary factor which might have contributed to the cytotoxicity of tyrosine in the above experiments with melanoma cultures is that quinones formed in the process of melanization are also toxic (see below). In the presence of glucose, the melanoma cell has an energy source to drive the synthesis of new glutathione to replace that which was removed via complexation with quinones during pheomelanin formation (§1.6). In actuality, the mechanism of toxicity may be a mixture of oxidative and quinone-mediated stress. Work by Graham et al. (1978a) with C-1300 neuroblastoma cultures (discussed below) demonstrated that L-3,4-DOPA, the first melanin intermediate generated from tyrosine, exerts its cytotoxic effect via the production of both oxy-radicals and toxic quinones.

The idea that toxic melanin intermediates could be used for treatment of melanoma was later explored by Pawelek et al. (1973). They demonstrated that 5 mM tyrosine killed melanotic (tyrosinase-containing) melanoma cells in culture but only decreased the growth rate of amelanotic (tyrosinase-lacking) clones of melanoma. Since the effect decreased when the medium was changed at more frequent intervals, it was suggested that toxic intermediates accumulated in the culture medium (Halaban and Lerner 1977a). This was supported by

experiments in which fibroblasts grown on coverslips were incubated with monolayer cultures of various clones of Cloudman melanoma cells in medium supplemented with 300 uM tyrosine (Halaban and Lerner 1977b). The rate of (^{3}H)-thymidine incorporation into fibroblast DNA was reduced in proportion to the tyrosinase activity present in the melanoma cultures. Furthermore, incubation of wild type Cloudman cells with tyrosine-supplemented medium which had previously been conditioned by clones of melanoma cells with various levels of tyrosinase activity, suppressed (^{3}H)-thymidine incorporation to a degree proportional to both the tyrosinase activity of the clones and the concentration of tyrosine in the medium.

L-3,4-DOPA, Dopamine, and Melanin Intermediates

The mechanism by which L-3,4-DOPA, the first melanin intermediate generated from tyrosine, exerts its cytotoxic effect was elucidated by Graham et al. (1978a) using C-1300 neuroblastoma cells. The mechanism was demonstrated to be a combination of oxy-radical- and quinone-mediated stress. Addition of 200 uM L-3,4-DOPA concurrently with (^{3}H)-thymidine inhibited incorporation of tritium label by 37% over a period of 3 hours. Norepinephrine, a scavenger of superoxide radical (Cohen and Heikkila 1977), decreased the inhibition of (^{3}H)-thymidine incorporation to 8 and 20%, when added at concentrations of 0.1 and 1 mM, respectively. For comparison, 1 mM norepinephrine decreased the inhibition of incorporation from 59 to 1% when tested with 50 uM 6-hydroxydopamine and from 57 to 0% when tested with 50 uM 6-hydroxy-DOPA; both 6-hydroxydopamine and 6-hydroxy-DOPA are potent generators

of oxy-radicals (§1.4). The quinone of L-3,4-DOPA was also shown to be a potent inhibitor of purified calf DNA polymerase-alpha, a sulfhydryl-dependent enzyme (155 uM L-3,4-DOPA-quinone inhibits by 50%, Graham et al. 1978a).

L-3,4-DOPA exerted an inhibitory effect upon growth of melanoma cultures (Wick et al. 1977a). Both melanotic (tyrosinase-containing) and amelanotic (tyrosinase-lacking) forms of melanoma were studied. When cultures were exposed to 6 mM L-3,4-DOPA for 1 hour, washed, and allowed to grow for an additional 48 hours, growth of S91A Cloudman melanoma (melanotic) was inhibited by 62%, and growth of human melanoma cells was inhibited by 35%. In contrast, the amelanotic S91B Cloudman melanoma was inhibited by only 5%, and L929 fibroblast and Chinese hamster ovary cells were unaffected. In subsequent studies, Wick (1980a) demonstrated that L-3,4-DOPA strongly inhibits DNA synthesis while only weakly inhibiting RNA or protein synthesis. Concurrent addition of 2 mM L-3,4-DOPA with radiolabeled precursor resulted in the reduction of 72% of the (^{3}H)-thymidine, 16% of the (^{3}H)-uridine, and 19% of the (^{3}H)-leucine incorporated into cells after one hour. This agrees with the finding that L-3,4-DOPA-quinone is a potent inhibitor of DNA polymerase-alpha. The 50% inhibitory concentration of preformed L-3,4-DOPA-quinone, purified by Sephadex G-25 chromatography at 4^{o}C., was reported to be 155 uM by Graham et al. (1978a), although Wick (1980a), using L-3,4-DOPA-quinone generated within the assay from L-3,4-DOPA and mushroom tyrosinase, reported a value of 3.8 uM. The lower concentration reported by Wick may reflect the formation of other products, in addition to L-3,4-DOPA-quinone, by

the action of mushroom tyrosinase. For example, Hansson et al. (1980) demonstrated that mushroom tyrosinase was capable of generating 5-hydroxy-DOPA from L-3,4-DOPA in addition to oxidizing the L-3,4-DOPA to L-3,4-DOPA-quinone. The 5-hydroxy-DOPA was also oxidized by mushroom tyrosinase to form fluorescent products (Agrup et al. 1982). In addition, other melanin intermediates and pre-melanin polymers can form in the presence of tyrosinase.

L-3,4-DOPA methyl ester and dopamine (Wick 1977b, 1978a, and 1980a) also selectively inhibited DNA synthesis in melanoma cultures. These compounds, in the presence of mushroom tyrosinase, were found to inhibit purified DNA polymerase-alpha in vitro, and both prodrugs were similar in potency to L-3,4-DOPA (Wick 1980a).

The efficacy of the melanin intermediates, dopachrome and 5,6-dihydroxyindole, have also been studied. Dopachrome was shown to be an inhibitor of DNA-polymerase-alpha (91 uM inhibits by 36%, Graham et al. 1978b). Pawelek and Lerner (1978) demonstrated a 50% inhibition of (^{3}H)-thymidine incorporation into Cloudman melanoma cells by 50 uM 5,6-dihydroxyindole, the precursor for indole-quinone. For comparison, 200 uM L-3,4-DOPA was required for the same effect.

In addition to inhibition of DNA polymerase-alpha, L-3,4-DOPA, L-3,4-DOPA methyl ester, and dopamine have also been reported to inhibit ribonucleotide reductase (Elford et al. 1981). Inhibition of ribonucleotide reductase would limit the supply of deoxyribonucleotides and correspondingly decrease DNA synthesis. The contribution of this effect to the toxicity directed against melanoma cells is presently under investigation by Fitzgerald and Wick (1983).

The life span of C57BL x DBA/2 mice bearing i.p. B-16 melanoma tumors increased by 34% following treatment with 600 mg L-3,4-DOPA methyl ester/kg, i.p., for 12 days beginning the day after tumor implantation (Wick 1977b). Extending the number of days of treatment with 600 mg L-3,4-DOPA methyl ester/kg to 24 resulted in an increased life span of 50%. Inclusion of Ro4-4602, a DOPA decarboxylase inhibitor, at 1g/kg to the regimen increased the potency of administered L-3,4-DOPA methyl ester so that a dose of 200 mg L-3,4-DOPA methyl ester/kg given i.p. for 12 days produced a 30% increase in life span. In subsequent in vivo studies, Wick et al. (1978b) demonstrated that incorporation of (^{3}H)-L-3,4-DOPA into S91A Cloudman melanoma was only 25% of the incorporation into adrenal tissue. Pretreatment of mice with Ro4-4602 increased the fraction of label incorporated into tumor tissue to 1.5-times that incorporated into adrenal tissue. These findings explain the increase in potency of L-3,4-DOPA methyl ester in vivo brought about by the addition of Ro4-4602 to the regimen. Although these results appear promising, it is not clear whether they can be used directly to predict the outcome of treatment of systemic metastases. Since the animals were injected i.p. and bore i.p. tumors, the tumor was exposed to higher concentrations of prodrug than were present systemically. However, the results should apply to the direct treatment of accessible tumors.

Clinical trials were conducted with dopamine as a tyrosinase-targeted prodrug (Wick 1982). Four patients with untreatable, advanced, metastatic melanoma received intravenous dopamine infusion at a rate of 20 ug/kg/min. Plasma levels of 31-52 uM dopamine were

obtained. Cardiovascular side effects, including increased blood pressure and heart rate, limited the dosage of dopamine. Two patients also complained of occipital headache. All side effects were reversible upon termination of treatment. In an effort to decrease the severity of cardiovascular side effects, the patients were given propranolol, 20 mg, every 4 hours. After 24 hours of infusion, the labeling index (percentage of cells actively synthesizing DNA) of biopsied tumor was found to be 8% (± 2 S.D., N=4) of the control value before treatment. These studies demonstrate that it is possible to virtually halt replication of melanoma cells in human subjects by employing the tyrosinase-activated prodrug, dopamine.

Gamma-Glutaminyl-4-Hydroxybenzene (GGHB)

The tyrosinase-activated prodrug, gamma-glutaminyl-4-hydroxybenzene (GGHB), occurs naturally in the gills of the common mushroom, Agaricus bisporus, and appears to function in the production of the dormant state during sporulation. GGHB is hydroxylated by mushroom tyrosinase to form gamma-glutaminyl-3,4-dihydroxybenzene. This compound is then oxidized by tyrosinase to yield gamma-glutaminyl-3,4-benzoquinone which is subsequently transformed non-enzymatically to a red compound, 490-quinone (Boekelheide et al. 1979).

490-Quinone, isolated from mushrooms, was demonstrated to be an inhibitor of the following purified dehydrogenases: succinate, xanthine, dihydrooratate, glyceraldehyde-3-phosphate, pyruvate, and alpha-ketoglutarate dehydrogenases (Weaver et al. 1968 and 1972). Purified DNA polymerase-alpha from L1210 leukemia cells was also

inhibited by 490-quinone (Graham et al. 1977). In later studies (Boekelheide et al. 1980a), the 490-quinone was identified as 2-hydroxy-4-imino-2,5-cyclohexadiene-1-one (i.e., 2-hydroxy-4-imino-quinone). 490-Quinone was found to be generated by either base-catalyzed hydrolysis of the amide bond of gamma-glutaminyl-3,4-dihydroxybenzene or by cleavage of the amide bond by gamma-glutamyl transpeptidase with subsequent autoxidation of the released 1-amino-3,4-dihydroxybenzene to 490-quinone.

B-16 tyrosinase hydroxylated GGHB and generated gamma-glutaminyl-3,4-dihydroxybenzene (Boekelheide et al. 1980b). However, the mammalian enzyme did not catalyze the oxidation of gamma-glutaminyl-3,4-dihydroxybenzene to its quinone form. Therefore, other routes by which GGHB could generate toxic species were investigated. It was demonstrated that gamma-glutamyl transpeptidase could cleave the amide bond of GGHB (Boekelheide et al. 1980b). The liberated 4-amino-phenol, or products generated from it by tyrosinase (viz., 490-quinone) may actually be the toxic species in mammalian systems. When 400 mg GGHB/kg was injected into C57BL/6J mice, destruction of melanocytes in hair follicles was apparent in histological sections (Burger et al. 1979); however, if the amide nitrogen of GGHB was blocked by introduction of an N-methyl group, the modified GGHB was no longer a substrate for gamma-glutamyl-transpeptidase and could not induce destruction of follicular melanocytes at doses of 400-1200 mg/kg (Boekelheide et al. 1980b). It was also demonstrated that injected 4-amino-phenol (50-400 mg/kg) produced destruction of follicular melanocytes in C57BL/6J mice (Boekelheide et al. 1980b).

These results are consistent with a proposed mechanism whereby GGHB is cleaved by gamma-glutamyl transpeptidase on the surface of the pigment cell to liberate 4-amino-phenol which might then be transformed to 490-quinone by tyrosinase within the cell. 4-Amino-phenol might also oxidize to generate an iminoquinone, which may also be toxic. Alternatively, mammalian tyrosinase may hydroxylate GGHB to form gamma-glutaminyl-3,4-dihydroxybenzene. Subsequent cleavage of the amide bond by gamma-glutamyl transpeptidase would then release the reduced form of 490-quinone (Boekelheide et al 1980a). Although all of these routes are possible, the actual metabolism of GGHB is unknown.

GGHB was tested against human melanoma (Kent clone) grown subcutaneously in either NIH Swiss or Sprague-Dawley nude mice (Vogel et al. 1970). In a preliminary trial, melanoma cells were preincubated for 3 hours with 4.8 mM GGHB before subcutaneous injection into NIH Swiss nude mice. After 28 days, the cells which had been pretreated with GGHB formed tumors only 40% as large as cells treated with diluent. In other trials, tumor-bearing mice received GGHB with or without probenecid, an agent which blocks secretion of organic acids by the kidney. Tumor-bearing NIH Swiss nude mice were treated with 400 mg GGHB/kg, 2-times daily, on days 1-7. After 21 days, the size of tumors in treated mice was 45% of control. Addition of 50 mg probenecid/kg 30 minutes before each GGHB injection increased the apparent potency; tumor size in treated mice was 19% of control. GGHB was also effective against human melanoma grown in Sprague-Dawley nude mice to a similar degree as when the tumors were grown in the NIH

Swiss mice.

1.3.2: Artificial Substrates as Tyrosinase-Activated Prodrugs

4-Hydroxyanisole

4-Hydroxyanisole, an analog of tyrosine, was extensively tested as a tyrosinase-activated prodrug. Riley (1969) demonstrated that 4-hydroxyanisole was a substrate for both mushroom tyrosinase and guinea pig epidermal tyrosinase. When cultures of guinea pig melanocytes were exposed to 4-hydroxyanisole (treatment varied from 5 nM for 24 hours to 5 mM for 30 minutes), blebbing of the cytoplasmic membranes to actual bursting of some melanocytes was detected by time-lapse photography (Riley 1970). The effect of 4-hydroxyanisole was dependent upon tyrosinase; for example, 100 uM diethyldithiocarbamate blocked the effect of 50 uM 4-hydroxyanisole (30 minute exposure) upon cultures of guinea pig melanocytes as monitored by light microscopy. In addition, the cytotoxicity of 10 mM or 5 mM 4-hydroxyanisole (30 minute exposure) could be blocked by the addition of 1 mM ascorbate. The blockade of cytotoxicity by ascorbate has important mechanistic ramifications. Both mammalian and mushroom tyrosinase hydroxylate 4-hydroxyanisole to form 3,4-dihydroxyanisole and subsequently, 3,4-dihydroxyanisole-quinone (Passi and Nazzaro-Porro 1981). If the quinone functions as an inhibitor of sulfhydryl enzymes, then ascorbate should protect against toxicity by maintaining the product predominantly in the reduced state. Therefore, it is likely that the cytotoxic effect is mediated via the production of toxic quinones.

When tested on CBA mice bearing subcutaneous Harding-Passey melanoma tumors, 4-hydroxyanisole produced a regression of the tumor (Dewey et al. 1977). Mice were given intratumor injections with 1.25 mg 5-hydroxyanisole in saline either twice a day on days 1-5 or once a day on days 1-5 and 8-12. Complete tumor regression was observed in 40% of the mice with no recurrence for at least 3 months. The authors stated that the drug was injected into the tumor to prevent rapid metabolic clearance which would otherwise necessitate the injection of much larger doses. Since the tumors were directly injected in these studies, it is not clear whether the results are directly applicable as a model for the treatment of systemic metastases. However, as discussed in the next paragraph, regional intraarterial perfusion with 4-hydroxyanisole proved useful in the treatment of patients with advanced melanoma. With regard to probable toxic effects at high doses, it was found by Riley (1969) that 5 mM 4-hydroxyanisole inhibited protein synthesis in HeLa cells by 56% and RNA synthesis by rat liver slices by 85% over a 1 hour period; neither HeLa cells nor rat liver contain tyrosinase. In addition, 0.5 mM 4-hydroxyanisole inhibited respiration almost completely in isolated rat liver mitochondria. Therefore, it appears that 4-hydroxyanisole is toxic to some tissues without prior activation by tyrosinase, and that the concern over systemic administration was well founded.

Even though the possibility of systemic toxicity was high, treatment with 4-hydroxyanisole in humans was still pursued (Morgan et al. 1981). Patients refractory to other means of treatment were given 4-hydroxyanisole by regional intraarterial infusion over a range of

total doses between 11-154 g/patient. In 4 out of 5 patients, tumors were observed to decrease in size, and treatment appeared to be well-tolerated with no evident side effects. In particular, no effect upon normal pigmentation was observed; therefore, there apparently exists a great enough differential in tyrosinase activity between tumor cells and normal, resting melanocytes to obtain a differential cytotoxic effect.

3,4-Dihydroxybenzylamine

Another agent which has been tested is 3,4-dihydroxybenzylamine (DHBA), an analog of dopamine. DHBA was found to exert its cytotoxic effect against melanoma cells by selectively inhibiting DNA synthesis (Wick 1980a). DNA polymerase-alpha was inhibited by DHBA (Wick 1980a, 1983). It was also reported (Elford et al. 1981) that DHBA inhibits ribonucleotide reductase. The contribution of this effect is currently under investigation by Wick et al. (1983).

When tested against melanoma cells in culture and in vivo, DHBA was more potent than dopamine, L-3,4-DOPA, or L-3,4-DOPA methyl ester (Wick 1979a). After 48 hours of exposure, the median inhibitory concentration of DHBA against B-16 melanoma cells was 0.21 mM. When tested against C57BL x DBA mice bearing intraperitoneal B-16 tumors, a dose of 600 mg DHBA/kg, given i.p. for 21 days, increased life span by 70% (Wick 1979a). Although it is an analog of dopamine, DBA lacked major sympathetic agonist activity and was less toxic than dopamine (Wick 1979a). Control mice injected daily for 7 days with 1 g DHBA/kg showed no decrease in survival. In comparison, the median survival

time of mice injected with 1 g dopamine/kg/day was 1.8 days (Wick 1979a). On the basis of potency and lack of side effects, DHBA was concluded to be better than L-3,4-DOPA, L-3,4-DOPA methyl ester, and dopamine as a tyrosinase-activated prodrug.

1.4: 6-HYDROXY-DOPA AND 6-HYDROXYDOPAMINE

6-Hydroxy-DOPA and 6-hydroxydopamine are well known neurotoxins (Kostrzewa and Jacobowitz 1974). Both compounds autoxidize at comparable rates at neutral pH with the production of quinones, H_2O_2, and superoxide radical (Heikkila and Cohen 1972, Heikkila and Cohen 1973, Cohen and Heikkila 1974, and Graham et al. 1978a); shown for 6-hydroxydopamine in equations (1) - (4). Reactions (1) and (2) describe the observed stoichiometry of the consumption of one mole of oxygen and the generation of one mole of H_2O_2 for each mole of 6-hydroxydopamine autoxidized. Reactions (3) and (4) constitute a self-propagating chain reaction catalyzed by superoxide radical. Since the rate of oxygen consumption by 6-hydroxydopamine was slower in the presence of superoxide dismutase, it was concluded that superoxide radical catalyzed autoxidation of 6-hydroxydopamine (Heikkila and Cohen 1973). These findings are consistent with the idea that reaction (1) is a rate-limiting step which can be by-passed by reaction (4).

(1) 6-Hydroxydopamine + O_2 = semiquinone + $O_2^{\bullet -}$

(2) semiquinone + $O_2^{\bullet -}$ = quinone + H_2O_2

(3) semiquinone + O_2 = quinone + $O_2^{\bullet -}$

(4) 6-Hydroxydopamine + $O_2^{\bullet -}$ = semiquinone + H_2O_2

The H_2O_2 and superoxide radical formed in the above reactions are believed to generate hydroxyl radical, a potent oxidizing species, in an iron-catalyzed Haber-Weiss reaction (Cohen 1978); equation (5).

(5) $$H_2O_2 + O_2^{\cdot-} \xrightarrow[Fe(II/III)]{} O_2 + OH^- + OH^{\cdot}$$

The toxicity of 6-hydroxy-DOPA and 6-hydroxydopamine has been attributed mainly to the oxy-radicals generated, rather than to the generation of quinones. Addition of norepinephrine, a scavenger of superoxide radical, to C-1300 neuroblastoma cultures completely blocked the toxicity of either 6-hydroxy-DOPA or 6-hydroxydopamine (Graham et al. 1978a). A similar phenomenon was previously noted by Sachs et al. (1975) in studies of the effects of 6-hydroxydopamine upon the adrenergic innervation of the rat iris. It was demonstrated that the toxicity of 6-hydroxydopamine, in vivo, could be amplified by depleting norepinephrine stores through the administration of alpha-methyl-p-tyrosine, an inhibitor of tyrosine hydroxylase. Furthermore, intraocularly injected norepinephrine blocked the neurotoxicity of 6-hydroxydopamine in the iris. In studies of the adrenergic innervation of the left atrium and of the iris in the mouse (Cohen et al. 1976), the toxicity of intraperitoneally administered 6-hydroxydopamine was blocked by various hydroxyl radical scavengers, such as 1-phenyl-3-(2-thiazolyl)-2-thiourea, diethyldithiocarbamate, methimazole, cysteamine, ethanol, and n-butanol. Perhaps the most convincing argument for the role of oxy-radicals, rather than quinones, as the toxic agents generated by 6-hydroxydopamine comes from experiments by Heikkila and Cohen (1972) which demonstrated that ascorbate amplified the damage caused by 6-hydroxydopamine in rat brain slices. If

quinones were the primary toxic species generated by 6-hydroxydopamine, then ascorbate would block toxicity by reducing the quinones as rapidly as they were formed. On the other hand, if the primary mode of toxicity was the generation of oxy-radicals, ascorbate would increase the toxicity of 6-hydroxydopamine by reducing the quinone and allowing further rounds of oxy-radical generation to occur.

6-Hydroxy-DOPA was approximately 100-times more potent against Cloudman melanoma cultures than L-3,4-DOPA (Wick et al. 1979b). In contrast to L-3,4-DOPA which acts by selectively inhibiting DNA synthesis, 6-hydroxy-DOPA depressed the synthesis of DNA, RNA, and protein. This is consistent with the production of a generalized metabolic insult which would occur if oxy-radicals were generated intracellularly and reacted non-specifically with macromolecules.

Although 6-hydroxy-DOPA proved to be potent against melanoma cultures, treatment of tumor-bearing C57BL x DBA mice was unsuccessful due to problems of systemic toxicity (Wick et al. 1979b). The systemic toxicity of 6-hydroxy-DOPA may have been due to the following factors: (1) All cells of the host were exposed to 6-hydroxy-DOPA, as well as to toxic products generated extracellularly. (2) It is likely that prolonged exposure to 6-Hydroxy-DOPA resulted in severe damage to central and peripheral catecholamine neurons.

1.5: CHARACTERISTICS OF TYROSINASE

Tyrosinase (monophenol, dihydroxyphenylalanine: oxygen oxidoreductase, EC 1.14.18.1) is a copper-containing enzyme which catalyzes the formation of melanin in mammals, plants, fungi, and bacteria. In addition, tyrosinase is involved in formation of the insect cuticle. In all forms of tyrosinase, the copper exists in a binuclear state within the active site and undergoes oxidation-reduction during the course of the catalytic cycle. Mushroom tyrosinase is a soluble enzyme which exists in multiple polymeric states. The predominant form is a tetramer, MW = 120,000, composed of 2 light subunits, MW = 13,400, and 2 heavy subunits, MW = 43,000. However, it is not known upon which subunits the 2 active site coppers reside. In contrast, mammalian tyrosinase exists as a membrane-bound monomer, MW = 66,700, and contains 2 equivalents of copper per monomer (Lerch 1981).

A simplified reaction scheme is presented in Figure 1-3 to illustrate the proposed reaction mechanism of the enzyme. Some of the evidence concerning points which also pertain to the present studies with 2,4-DOPA will be summarized here; however, a thorough discussion of the experimental evidence concerning other aspects can be found in the excellent review on the structure and mechanism of tyrosinase written by Lerch (1981). Tyrosinase, via the monophenolase reaction (middle and lower sequences), converts L-tyrosine to L-3,4-DOPA with the concomitant oxidation of the active site copper from 1^+ to 2^+. Subsequently, L-3,4-DOPA is oxidized to its quinone form, and the copper is reduced to 1^+ in the process. The

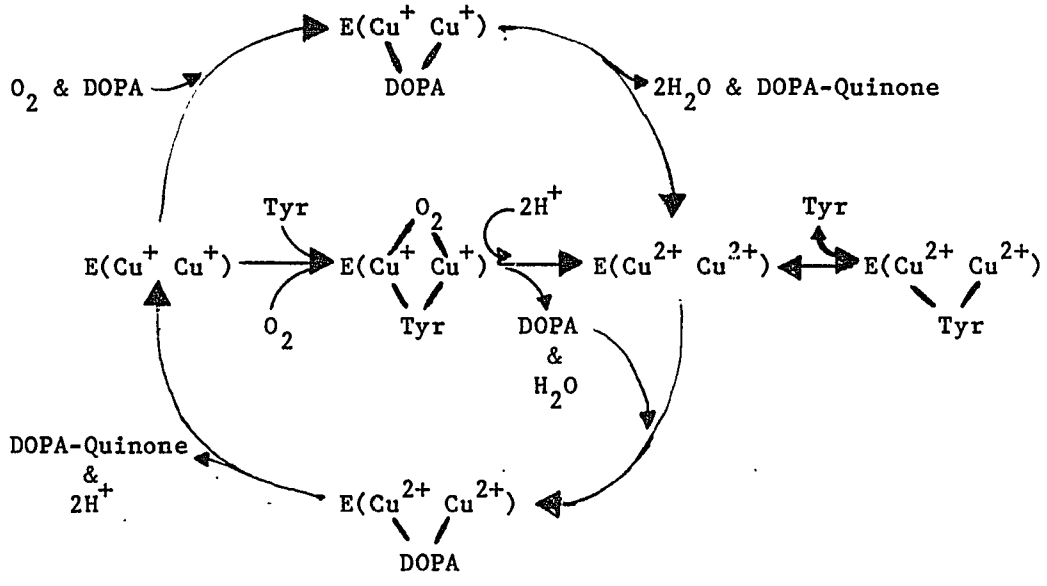


Figure 1-3. Mechanism of Tyrosinase

E = Tyrosinase (2 coppers per active site)

Tyr = Tyrosine

DOPA = L-3,4-DOPA

"resting" form of tyrosinase isolated from the common mushroom, Agaricus bisporus, contains predominantly antiferromagnetically coupled Cu^{2+} (Makino et al. 1974). Resting tyrosinase can combine with H_2O_2 to form "oxy-tyrosinase" which is equivalent to enzyme containing Cu^{1+} complexed to oxygen (Jolley et al. 1974). Oxy-tyrosinase proved to be a valuable tool for kinetic studies. Makino and Mason (1973) suggested that the enzyme must be in the reduced form to hydroxylate substrates based upon the demonstration that oxy-tyrosinase catalyzed the hydroxylation of substrates at rates similar to those found when the overall reaction was monitored. The initial rate of disappearance of oxy-tyrosinase was monitored spectroscopically (A_{345}), and the overall rate of reaction of substrates with tyrosinase was determined by monitoring oxygen consumption. Makino et al. (1974) further demonstrated that the Cu^{2+} in resting tyrosinase could be reduced to Cu^{1+} by anaerobic incubation with t-butyl-catechol, an analog of L-3,4-DOPA. The number of electrons transferred was monitored by anaerobic titration of the oxidized t-butyl-o-quinone. It was demonstrated by Pomerantz (1966) in studies of hamster melanoma tyrosinase that L-tyrosine at concentrations greater than 800 uM significantly inhibits the monophenolase reaction (K_I = 300 uM). The inhibition could be reversed by elevation of the L-3,4-DOPA concentration, suggesting that the two compounds compete for the oxidized form of tyrosinase (Figure 1-3, right side). L-3,4-DOPA was also found to be a competitive inhibitor (K_I = 400 uM) of the hydroxylation of L-tyrosine by hamster melanoma tyrosinase (Pomerantz 1966); this is consistent with the

reduced form of tyrosinase being able to combine with either L-tyrosine or L-3,4-DOPA (Figure 1-3, left side).

The diphenolase activity of tyrosinase involves the oxidation of two equivalents of L-3,4-DOPA per cycle and the reduction of 1 mole of molecular oxygen to 2 moles of water (upper and lower sequences, Figure 1-3). From the stoichiometry of the reaction, and the competition experiments of Pomerantz (1966), the upper loop in Figure 1-3 can be inferred. Evidence for the lower loop was discussed above.

L-3,4-DOPA has been shown to function as cofactor ($K_{activator}$ = 2 uM) for mammalian tyrosinase and is present at 20-40 uM in homogenates of hamster melanoma tissue (Pomerantz and Warner 1967). In experiments with mushroom tyrosinase, Duckworth and Coleman (1970) also observed that L-3,4-DOPA functioned as a cofactor ($K_{activator}$ = 0.5 uM). L-Tyrosine competed with the cofactor activity of L-3,4-DOPA; the $K_{activator}$ increased when L-tyrosine concentration was increased. These observations are consistent with either of two hypotheses. L-3,4-DOPA may act by binding to an allosteric "activator" site, or L-3,4-DOPA may function simply as a source of reducing equivalents (lower loop in Figure 1-3). In experiments with Streptomyces tyrosinase (Lerch and Ettlinger 1972), 3,4-dihydroxyphenylproprionic acid and 3,4-dihydroxybenzoic acid were tested as cosubstrate for the tyrosinase-catalyzed hydroxylation of p-hydroxyphenylproprionic acid. Both diphenols had similar K_m values for oxidation by tyrosinase (1.055 and 1.023 mM, respectively), but their k_{cat} values differed greatly (1830 and 8.2 sec^{-1}, respectively). The ability of the diphenols to function as activators was found to be

directly proportional to their rates of oxidation (i.e., k_{cat}); the $K_{activator}$ was 0.85 uM for 3,4-dihydroxyphenylproprionic acid and 165 uM for 3,4-dihydroxybenzoic acid. This is consistent with the concept that the cofactor acts as a source of reducing equivalents for the monophenolase reaction. Consequently, the monophenolase activity should be considered to be a mixed-function oxidase activity.

1.6: SYNTHESIS OF MELANIN

Tyrosinase, which is responsible for the generation of melanin pigment, is synthesized in the rough endoplasmic reticulum of melanocytes and packaged into premelanosomes by the Golgi apparatus. The premelanosomes are the site of melanin formation, and they subsequently mature into melanin-containing melanosomes in which no tyrosinase activity is detectable. The mature melanosomes are then transported to the dendrites of the melanocytes, phagocytized by the keratinocytes of the epidermis, and transported to the supranuclear region (i.e., between the nucleus and the skin surface) of the keratinocyte (Breathnach 1969).

The reactions leading to the formation of melanin are shown in Figure 1-1. Tyrosinase has been shown to catalyze three of the reactions involved in the process of melanogenesis (Körner and Pawelek 1982). Tyrosinase catalyzes: (1) the conversion of L-tyrosine to L-3,4-DOPA, (2) the oxidation of L-3,4-DOPA to L-3,4-DOPA-quinone, and (3) the oxidation of 5,6-dihydroxyindole to indole-5,6-quinone. L-3,4-DOPA is required as a cosubstrate for reactions (1) and (3). In both reactions (1) and (2), L-tyrosine and L-3,4-DOPA show competitive

inhibition with one another (§1.5). Since the inhibition was competitive, it appears that L-tyrosine and L-3,4-DOPA bind to the same active site on tyrosinase. Reaction (3) is unusual in that L-tyrosine noncompetitively inhibits the oxidation of 5,6-dihydroxyindole (Körner and Pawelek 1982). Since the inhibition was non-competitive, the authors suggested that 5,6-dihydroxyindole binds to a different active site than L-tyrosine or L-3,4-DOPA.

Körner and Pawelek (1980) discovered a heat stable, low molecular weight factor in homogenates of melanoma tissue which catalyzed the conversion of dopachrome to 5,6-dihydroxyindole-2-carboxylic acid. They named the molecule "dopachrome conversion factor" (DCF). The role of DCF in control of melanogenesis is presently unknown.

In nature, two basic types of melanin can be found, dark brown eumelanin and yellow to red pheomelanin. The difference between the two types of melanin is the high concentration of sulfur found in pheomelanins. It is thought that the sulfur originates from the condensation of glutathione or cysteine with L-3,4-DOPA-quinone, which is subsequently incorporated into melanin (Pawelek and Körner 1982). In support of this hypothesis, both cysteinyl-DOPA (Rorsman et al. 1973) and glutathionyl-DOPA (Agrup et al. 1977) have been isolated from human melanoma tissue. Agrup et al. (1975) also demonstrated that homogenates of melanoma tissue were capable of transforming glutathionyl-DOPA to cysteinyl-DOPA. The discovery of 5-S-Cysteinyl-DOPA is clinically important, since it is elevated in the serum of patients with melanoma (Agrup et al. 1979, Marnot et al. 1983) and can be easily monitored by HPLC (Hansson et al. 1978).

A minor route in the pathway of melanogenesis was discovered by Hansson et al. (1980). It was demonstrated that mushroom tyrosinase hydroxylated L-3,4-DOPA in the 5-position to generate "5-hydroxy-DOPA." Both the 5- and 3-positions are meta to the side chain. Apparently, the specificity of mushroom tyrosinase is such that it is the meta position of the substrate, not just the 3-position, which is subject to hydroxylation. In later studies, Agrup et al. (1982) demonstrated that the rate of synthesis of 5-hydroxy-DOPA from L-3,4-DOPA was an order of magnitude lower than the rate of oxidation of L-3,4-DOPA to dopachrome. 5-Hydroxy-DOPA was also found to be a substrate for mushroom tyrosinase and was oxidized to a stable fluorophore (excitation maximum at 425 nm, emission maximum at 500 nm).

1.7: REGULATION OF MELANOGENESIS

One of the most apparent effects of exposure to ultraviolet radiation is tanning of the skin. Evidence has been presented by Pavlovitch et al. (1982) that tanning of the skin may serve as a natural mechanism to regulate the amount of vitamin D_3 formed by ultraviolet photolysis of 7-dehydrocholesterol. Brown Norway rats, fed a vitamin D-deficient diet or a diet supplemented with 30 IU of vitamin D/day, were exposed to 0.1 joules/cm^2 of UV irradiation (120-320 nm). One day later, tyrosinase activity present in skin biopsies was approximately 1.8-times and 2.8-times pre-exposure levels, respectively. The smaller increase in tyrosinase activity in the vitamin D-deficient group was not due to low serum calcium. Rats fed

a vitamin D-deficient diet supplemented with calcium maintained the same serum calcium levels as rats fed the vitamin D-supplemented diet, but did not exhibit a greater increase in tyrosinase activity after UV irradiation when compared to rats fed a vitamin D-deficient diet without supplemental calcium. In all three groups, the UV induced stimulation of tyrosinase activity was preceded by an increase in cAMP, but the increase was greater in rats fed the vitamin D-supplemented diet. The authors concluded that the synthesis of vitamin D results in the activation of tyrosinase, probably via stimulation of cAMP synthesis (see below), with the subsequent formation of melanin pigment. Since melanin is known to absorb UV light, the authors speculated that an increase in melanin content within the skin will result in a decrease in vitamin D synthesis through UV photolysis; thus, a feedback loop may be present within the epidermis to control the synthesis of vitamin D.

Melanocyte stimulating hormone (MSH) has also been shown to exert a positive regulatory effect upon tyrosinase activity. MSH stimulated tyrosinase activity in Cloudman melanoma cultures, but only when cells were in the G2 phase of the growth cycle (Wong et al. 1974). In agreement with earlier observations that dibutyryl-cAMP increased the melanization of cultured Cloudman melanoma cells (Johnson and Pastan 1972), Wong et al. found that the increase in tyrosinase activity paralleled an increase in intracellular cAMP. Later studies (Varga et al. 1974) revealed that ^{125}I-MSH was bound to Cloudman melanoma cells maximally and stimulated tyrosinase activity only when the cells were in the G2 phase of growth; however, dibutyryl-cAMP was found to

stimulate tyrosinase activity independent of the phase of growth. These results suggest that MSH binds to receptors on cells during the G2 growth phase and stimulates tyrosinase activity by elevating the level of cAMP.

The mechanism by which MSH activates tyrosinase was further explored by Wong and Pawelek (1975). When Cloudman melanoma cultures were incubated with (^{3}H)-leucine plus or minus MSH, there was no difference in the amount of label incorporated into the soluble fraction of tyrosinase. Neither cycloheximide (inhibits protein synthesis) nor actinomycin D (inhibits RNA synthesis) blocked the action of MSH. These results indicate that MSH did not increase the synthetic rate of new molecules of tyrosinase. Furthermore, when cells were grown in medium with or without MSH, and then exposed to cycloheximide to prevent protein synthesis, the half life of tyrosinase was unaffected by MSH. However, cultures which had not been treated with MSH did contain an inhibitor of tyrosinase; extracts of cells grown in the absence of MSH inhibited tyrosinase purified from MSH-treated cells by 90%. The authors proposed that MSH may function to promote the inactivation of a naturally occurring tyrosinase inhibitor, thereby resulting in activation of tyrosinase.

The mechanism by which MSH inactivates the natural inhibitor of tyrosinase was explored by Körner and Pawelek (1977). Data were presented which indicated that cAMP activates a protein kinase which in turn phosphorylates the tyrosinase inhibitor, thereby rendering the inhibitor inactive against tyrosinase. In a cell free system derived from Cloudman melanoma cells, tyrosinase activity was stimulated by

the addition of cAMP, ATP, and kinase; the kinase added was a cAMP-dependent protein kinase isolated from Cloudman melanoma cultures. Activation of tyrosinase did not occur when kinase was ommitted from the reaction mixture. Furthermore, the addition of calf brain kinase modulator, an inhibitor of protein kinases, blocked the activation of tyrosinase. A heat-sensitive phosphatase activity was also demonstrated in extracts of Cloudman melanoma cells. The phosphatase was active against ^{32}P-labeled proteins prepared by incubation of melanoma cell extracts with cAMP, ^{32}P-ATP, and kinase. The phosphatase was shown to be different from the tyrosinase inhibitor, which is heat-stable. Although the authors had not isolated enough phosphatase activity to completely analyze its effect upon tyrosinase activation, they did demonstrate that the activation of tyrosinase by added cAMP, ATP, and kinase in a cell free system could be blocked by the addition of calf intestine alkaline phosphatase. This suggests that tyrosinase activity is also under negative control via the action of the phosphatase present within the melanoma cell.

The tyrosinase activity of melanoma cells has been shown to be regulated by other factors not dependent upon cAMP. Wade and Burkart (1978) demonstrated that tyrosinase activity in B-16 melanoma cultures increased slowly during logarithmic growth and reached a peak shortly after the cultures reached confluence. Thereafter, the tyrosinase activity began to slowly return to initial levels. There was no correlation between the level of tyrosinase activity and the level of cAMP.

Retinoic acid also increased tyrosinase activity without altering

the level of cAMP (Lotan and Lotan 1981). However, the mechanism may be related to the proposed mechanism for MSH discussed above. It has been reported that retinoic acid enhanced the activity of cAMP dependent protein kinase in cultures of B16 melanoma (Niles and Logue 1979). It may therefore be the case that the tyrosinase regulatory system, demonstrated by Körner and Pawelek (1977), is sensitized to normal levels of cAMP by the administration of retinoic acid.

Chapter 2: METHODS

2.1: ENZYMATIC STUDIES

2.1.1: Enzyme Sources

Mushroom tyrosinase was obtained commercially from Sigma and used without further purification.

B-16 tyrosinase was obtained from tumor tissues grown in vivo. C57BL/6J mice were injected subcutaneously with 10^6 B-16 melanoma cells in a volume of 0.2 mL of Ham's F-10 (containing 10% fetal bovine serum). After the tumors had grown to an area of approximately 1 cm^2, hosts were killed by cervical dislocation, and tumors removed and stored on ice. Tumor tissue was homogenized in 9 parts of 50 mM phosphate buffer, pH 6.8, containing 0.1% sodium cholate, and stirred for an additional 30 minutes. Sodium cholate, a membrane solubilizing agent, was included to keep the normally membrane-bound mammalian tyrosinase in solution. The enzyme was enriched by ammonium sulfate fractionation and separated from insoluble melanin and soluble melanin intermediates which might act to inhibit tyrosinase activity. The homogenate was brought to 40% saturation in ammonium sulfate and centrifuged at 700 g for 40 minutes. The pellet consisted predominantly of jet-black, insoluble melanin, and was discarded. The supernatant was then brought to 60% saturation in ammonium sulfate and centrifuged at 700 g for 40 minutes to yield a pellet enriched in tyrosinase activity. At this point, the pellet was still contaminated with black melanin pigment. Melanin was removed by redissolving the

pellet in fresh buffer and centrifuging at 700 g for 40 minutes. The insoluble melanin formed a pellet at the bottom of the tube. The supernatant, containing tyrosinase, was then decanted. Enzyme was reprecipitated by making the solution 80% saturated in ammonium sulfate. The enzyme pellet was again redissolved, centrifuged free of any remaining insoluble melanin, and reprecipitated to to yield a dull gray, flocculent precipitate. During the purification, tyrosinase activity was monitored with the dopachrome method (Pomerantz 1963). Briefly, enzyme is added to 2.5 mM L-3,4-DOPA in 50 mM phosphate buffer, pH 6.8, and the formation of dopachrome is monitored via its absorbance at 475 nm.

In some experiments, the homogenate was used without further purification so that the reaction rate of tyrosinase with 2,4-DOPA could be measured using a known dilution of the tumor cell material. The 40-60% ammonium sulfate fraction was used for experiments in which kinetic parameters were determined.

2.1.2: HPLC Characterization of Products

Products were generated at 22°C by addition of mushroom tyrosinase to a solution containing a final concentration of 1 mM substrate (2,4-DOPA or 2,4-dopamine), 50 uM L-3,4-DOPA (cosubstrate), 1 mM ascorbate (maintains the product in the reduced state), and 100 uM DTPA (metal chelator which suppresses the metal-catalyzed autoxidation of ascorbate or product) in 50 mM phosphate buffer, pH 6.8.

When B-16 tyrosinase was used, the solution contained 0.1% sodium

cholate to solubilize the enzyme. The ascorbate concentration was initially 1 mM when B-16 tyrosinase was used and was decreased to 0.1 mM in later experiments. All other components were present as above.

High performance liquid chromatography (HPLC, Waters) with electrochemical detection (Bioanalytical Systems) was utilized to identify reaction products. A 10 um bead C-18 microBondapak column (Waters), 25 cm in length, was first used and was replaced in later experiments by a 5 um bead C-18 column (Altex), 25 cm in length.

6-Hydroxy-DOPA, L-3,4-DOPA, 2,4-DOPA, and ascorbate were separated by a mobile phase of 50 mM phosphate buffer, pH 3.0, containing 1 mM EDTA at flow rates of 0.5 to 1.5 mL/minute (depending upon the efficiency of the column). Separation of 6-hydroxydopamine from L-3,4-DOPA, 2,4-dopamine, 2,3,4- and 2,4,6-trihydroxyphenylethylamine, and ascorbate was accomplished with a mobile phase of 50 mM acetate buffer, pH 5.1, containing 1 mM EDTA, 2.5 mM sodium octyl sulfate, and 20% methanol at a flow rate of 2.0 mL/minute.

The electrochemical detector was usually set at +0.9 volts versus a Ag/AgCl reference electrode for routine analyses. At this setting, the products, substrates, L-3,4-DOPA, and ascorbate all gave rise to peaks on the chromatogram. In initial experiments with B-16 tyrosinase, approximately 1/16 the amount of enzyme was used for each sample compared to experiments with mushroom tyrosinase. Since the amount of product generated was proportionately less, the sensitivity of the detector was increased correspondingly. Ascorbate was initially used at a concentration of 1 mM. Since the 6-hydroxy-DOPA peak rides on the tail of the ascorbate peak (see ahead, Figure 3-1),

it was not possible to analyze 6-hydroxy-DOPA at a potential of +0.9 volts; the tail of the ascorbate peak was offscale when the detector was set at the sensitivity necessary to see product. To alleviate the problem, the detector was set at +0.4 volts; in this way, the ascorbate peak was diminished sufficiently to allow quantitation of 6-hydroxy-DOPA. In later experiments with B-16 tyrosinase in which it was necessary to analyze product at higher electrode potentials, the ascorbate concentration was reduced to 0.1 mM, and B-16 tyrosinase was added at approximately 25-33% of the concentrations used for experiments with mushroom tyrosinase.

To determine if the products would co-chromatograph with standards, both product and standard were loaded into an HPLC syringe, and the mixture injected. The resultant peak was compared to an injection of standard alone. The compounds were considered to co-chromatograph if the mixture of product and standard produced a clean peak similar in shape and identical in retention time to the peak produced by the product alone.

Samples were analyzed by HPLC with the electrode set at various potentials to determine the electrochemical properties of the products and standards. The peak height generated by a sample is proportional to the amount of sample oxidized in the thin layer of mobile phase at the electrode surface (i.e., the detector is a precision ammeter). When all other factors are held constant, changing the electrode potential will cause the relative peak height to vary in a manner dependent upon the oxidizability of the sample. Compounds which are easily oxidized (such as 6-hydroxy-DOPA) will produce significant

peak heights at relatively low voltages. On the other hand, a compound which is less readily oxidized (such as L-3,4-DOPA) may not produce a detectable peak at the same voltage. As the voltage is increased, the amount of sample oxidized at the electrode surface increases until a point is reached at which virtually all of the sample is oxidized within the thin layer immediately adjacent to the electrode surface. The resultant plot of peak height versus potential is a smooth curve which rises and then plateaus (Bioanalytical Systems LC-4B Reference Manual, 1982). The potential at which the peak height is half-maximal is defined as the half-wave potential and is a characteristic of the individual compound. An easily oxidized compound has a low half-wave potential, and a relatively stable compound has a high half-wave potential.

Products were quantitated by comparison with calibration standards at a constant electrode potential. A sample calibration curve for 6-hydroxy-DOPA is shown in Figure 2-1. At least two concentrations of standard were carried in each experiment. The concentrations of standards were chosen so as to bracket the concentration range of generated products.

2.1.3: Determination of Kinetic Parameters and pH Dependence

K_m and V_{max} were determined by varying the substrate concentration while utilizing all other components at constant concentration. All reactions were carried out at 22°C. The components of the standard reaction mixture were substrate (2,4-DOPA or 2,4-dopamine), 1 mM ascorbate, 50 uM L-3,4-DOPA, and 100 uM DTPA in

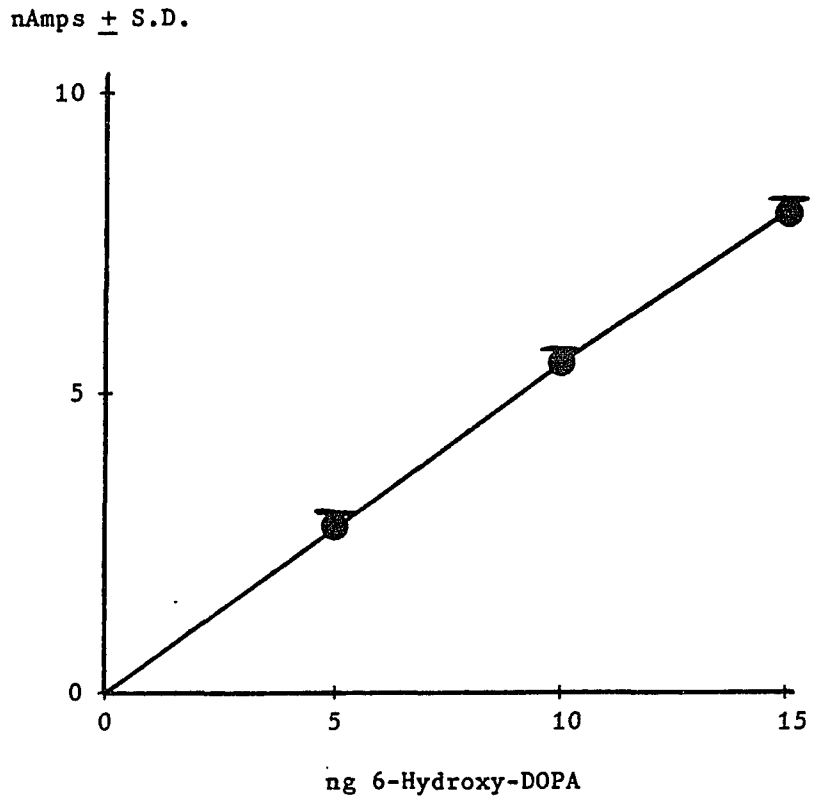


Figure 2-1. 6-Hydroxy-DOPA Calibration Curve

6-Hydroxy-DOPA was analyzed by HPLC with electrochemical detection. A mobile phase consisting of 50 mM phosphate buffer, pH 3.0, containing 1 mM EDTA was used at a flow rate of 1.5 mL/minute. The detector electrode potential was set at +0.8 volts (versus a Ag/AgCl reference electrode). Each point represents the mean of duplicate injections.

50 mM phosphate, pH 6.8. The reaction was initiated by adding mushroom tyrosinase (see individual experiments for the number of units added), in 50 mM phosphate buffer, pH 6.8, to the reaction mixture. When B-16 tyrosinase was used, the ascorbate level was 0.1 mM, and 0.1% sodium cholate was present in the buffer; all other components were present as listed above. Aliquots of 0.45 mL were removed at either 2, 4, and 6 minutes (mushroom tyrosinase) or 2, 6, and 10 minutes (B-16 tyrosinase) and the reaction stopped by mixing with 50 uL of 4N PCA. Controls contained either boiled enzyme or buffer in place of enzyme and were sampled before and after the reaction period. Samples were centrifuged at 8,700 g for 1 minute in a Beckman microfuge and stored on ice until analyzed for product by HPLC. The data were analyzed by the method of Lineweaver and Burk.

$K_{activator}$ was determined by varying the L-3,4-DOPA concentration while holding all other concentrations constant. The ascorbate concentration was 1 mM when mushroom tyrosinase was used and 0.1 mM when B-16 tyrosinase was used. The substrate concentration was 1 mM. The reaction was carried out at 22°C in 50 mM phosphate buffer, pH 6.8, containing 100 uM DTPA; 0.1% sodium cholate was also present when B-16 tyrosinase was used. Data were analyzed by the method of Lineweaver and Burk.

The pH dependence of the reactions with mushroom tyrosinase was determined using various buffers. Acetate was used to buffer at pH 5.5, phosphate at pH 6.8 - 7.7, pyrophosphate at pH 8.3, TRIS at pH 8.6 - 9.1, and carbonate at pH 9.6 - 10. Mushroom tyrosinase in 1 mM phosphate buffer, pH 6.8, was added to a solution of 1 mM substrate,

30 uM L-3,4-DOPA, 1 mM ascorbate, and 100 uM DTPA in the appropriate buffer at 22°C. The pH of the solution was checked at the end of the reaction period to monitor for any change brought about by the stock enzyme buffer (ranges given above are already adjusted for this effect).

When B-16 tyrosinase was studied, ascorbate was present at 0.1 mM, and the buffer contained 0.1% sodium cholate. Acetate was used to buffer at pH 5.6, phosphate at pH 6.8 - 7.6, pyrophosphate at pH 8.4, and carbonate at pH 9.0 (note: the original carbonate buffer was pH 10.0, but the pH dropped to 9.0 upon the addition of enzyme stock).

2.2: CELL CULTURE METHODS

2.2.1: Cell Cultures and Growth Media

Monolayer cultures of mouse B-16 melanoma cells were routinely maintained at 37°C in a moist 5% CO_2 atmosphere. The cultures used in initial studies were designated B-16_1 and were a mixture of melanized and non-melanized cells. The growth medium used for these B-16_1 cultures was Gibco minimal essential medium (MEM) with D-valine, 20% calf serum, 100 units penicillin G/mL, and 100 ug streptomycin/mL, pH 7.3. After approximately 16 months in the above growth medium, the cultures began to rapidly lose their melanized phenotype. Eventually, they became completely amelanotic and were not useful for studies.

A new B-16 melanoma culture, designated B-16_2, was established from tumor tissue which was aseptically removed from a C57BL/6J mouse. Tumor tissue was rinsed in sterile growth medium, finely minced with dissection scissors, and inoculated into new flasks with sufficient media to just cover the tumor pieces. The growth medium was changed daily. Within two weeks, the cells had grown out from the explants to form a confluent monolayer and were used to initiate a new cell line.

It was suspected that the loss of melanized phenotype was related to properties of the growth medium, since new cultures became more intensely melanized when 10% fetal bovine serum was substituted for 20% calf serum. Furthermore, Pawelek (1979) has suggested that melanoma cells should be maintained in medium low in tyrosine. Tyrosine is the natural precursor of melanin and has been shown to exert toxicity against melanoma in vitro through the formation of

toxic melanin intermediates (Pawelek 1973, Halaban and Lerner 1977a and 1977b). MEM contains 200 uM tyrosine, whereas Ham's F-10 medium contains only 10 uM tyrosine and has been recommended by other investigators (Pawelek 1979). Consequently, the new B-16 cell culture, $B\text{-}16_2$, was maintained in Ham's F-10 containing 10% fetal bovine serum and antibiotics. As of the time of this writing, the new cultures have maintained a subpopulation of melanized cells for 22 months.

Cloudman mouse melanoma cells were grown as monolayers in Ham's F-10 containing 7.5% horse serum, 2.5% fetal bovine serum, and antibiotics.

The following cell types and media were used in control experiments: monolayer cultures of mouse MJY-alpha mammary tumor cells were grown in RPMI 1640 containing 18% fetal bovine serum, 10 ug insulin/mL, and antibiotics. Mouse C-1300 neuroblastoma cells were grown as monolayer cultures in Ham's F-10 containing 10% fetal bovine serum and antibiotics. Suspensions of mouse L-1210 leukemia cells were grown in RPMI 1640 containing 18% fetal bovine serum and antibiotics. All growth media were changed daily.

2.2.2: Passage of Cell Cultures

Cells were released from monolayer cultures by incubation for 5 to 10 minutes with 0.1% trypsin/ 0.05% EDTA in Hank's balanced salt solution without calcium or magnesium. The resultant cell suspension was added to an equal volume of medium and centrifuged at 700 g for 10 minutes. The cell pellet was resuspended in fresh medium, and an

aliquot was sterilely removed for counting on a hemocytometer. Viability was assessed by exclusion of the vital dye, trypan blue. Cells were then seeded in new flasks (usually 40,000 cells per cm^2).

L-1210 suspensions were pelleted, resuspended in fresh medium, counted, and aliquoted into new flasks at a density of 100,000 per mL. All cultures were passaged every 7 to 10 days with the exception of L-1210 cultures which were passaged every 3-4 days.

2.2.3: Experimental Procedures

Treatment with 2,4-DOPA: Effect upon Cell Survival

2,4-DOPA was dissolved in fresh growth media at 0, 1, 2.5, 3, or 5 mM. All solutions were sterilized by filtration through a 0.22 um Millex GS filter attached to a syringe. 2,4-DOPA-containing media was added to monolayer cultures in place of regular growth media. All media were changed daily. To treat the L-1210 suspension cultures, the cells were first pelleted, then one-half of the medium was exchanged for medium containing 2,4-DOPA. The medium was exchanged daily.

Cells from replicate monolayer cultures were released by incubation with trypsin/EDTA and counted as described above (§2.2.2). Viable cells were not observed in the supernatant media of treated or control B-16 melanoma, Cloudman melanoma cultures, or MJY-alpha cultures. However, neuroblastoma cultures did release viable cells into the supernatant medium in both treated and control cultures. Consequently, it was necessary to count both the supernatant and the

monolayer. The supernatant (0.5 mL) was removed, and the monolayers released by addition of 0.5 mL of trypsin/EDTA. Equal parts of the monolayer suspension, supernatant, and trypan blue were mixed and loaded onto a hemocytometer for counting. L-1210 suspensions were aliquoted into trypan blue and counted.

Histochemical Staining of Tyrosinase

Cells were stained for tyrosinase activity by a modification of the method of Lillie (1956a). The method entailed lightly fixing the cells to preserve the architecture, followed by an overnight incubation in a solution of 1 mg L-3,4-DOPA/mL at pH 7.0. The L-3,4-DOPA served as a substrate for tyrosinase and was converted to melanin within the cell. Cells that are only lightly melanized in vitro then appear as heavily melanized after the staining procedure has been performed. Enhancement of the natural melanization of cells was useful when preparing photomicrographs or when assessing the percentage of melanized cells in the total population of cells.

B-16 or Cloudman melanoma cultures were released from replicate monolayer cultures by incubation with trypsin/EDTA and centrifuged for 10 minutes at 700 g. The pellet was resuspended in one drop of medium and lightly fixed in suspension with either 4% formaldehyde containing 2 gram% calcium acetate, 10 minute fixation time (Tables 4-1 and 4-2 and Figure 4-2), or 3% glutaraldehyde in 10 mM phosphate buffered saline (PBS), pH 7.0, 2 minute fixation time (all other experiments). (This latter procedure was adopted since a better resolution of stain versus background was obtained. It was also noted that inclusion of

100 uM DTPA in the L-3,4-DOPA staining solution also decreased background to a small extent). Cells were then immediately centrifuged for 10 minutes at 700 g, washed in PBS, and pelleted again. Cell pellets were then resuspended in a solution of 1 mg/mL L-3,4-DOPA in PBS. Following incubation for 24 hours at room temperature, the cells were pelleted, washed in PBS, pelleted, and resuspended in PBS for scoring. The suspensions were loaded onto a hemocytometer and viewed at 200 x magnification. Cells scored as melanized contained darkly stained melanosomes which were visible as dark granules. Non-melanized cells contained no visibly stained granules and exhibited a diffuse light brown background color. The background color was probably due to L-3,4-DOPA autoxidation and subsequent non-enzymatic formation of melanin; aliquots of the L-3,4-DOPA staining solution which were left standing overnight in the absence of cells exhibited the same light brown background color. The fraction of cells scored as melanized was used to weight the raw cell counts to calculate the number of melanized and non-melanized cells per culture. Since trypsin has been demonstrated to digest dead cells (Hoskins et al. 1956), the cell counts determined by this method represent viable melanized and non-melanized cells.

Cultures were prepared for photomicroscopy by growing cells on glass coverslips. Cells were fixed and stained as described above, counter-stained with Nile blue (Lillie 1956b) which stains melanin green, and nuclei and cytoplasm blue, and then mounted in glycerol on glass slides.

Quantitation of Tyrosinase Activity

Tyrosinase activity was measured by a modification of the method of Wick et al. (1979b) which measures tyrosinase-catalyzed incorporation of L-3,4-DOPA into melanin. Since all of the hydrogen atoms of L-3,4-DOPA can potentially be exchanged at one point or another during melanin synthesis (Farishian and Whittaker, 1979), (^{14}C)-D,L-3,4-DOPA (1 uCi/0.5 mL, 18 uM) was used in place of (^{3}H)-L-3,4,-DOPA. Before measuring tyrosinase activity, the cultures were washed 2-times with balanced salt solution and then allowed to incubate for 30 minutes in balanced salt solution to allow efflux of intracellular 2,4-DOPA. Briefly, the procedure was to label the cultures for 1 hour with (^{14}C)-D,L-3,4-DOPA, wash away excess label, precipitate with 10% TCA, wash, and dissolve in 1 N KOH for scintillation counting.

Assessment of the Content of Prelabeled (^{14}C)-Melanin in B-16$_2$ Cultures Treated with 2,4-DOPA

B-16$_2$ cells, grown as replicate monolayer cultures in 2-cm^2 culture wells, were labeled with (^{14}C)-D,L-DOPA 2 days prior to the initiation of 2,4-DOPA treatment. Cultures were washed 2-times with balanced salt solution (Hank's balanced salt solution buffered with HEPES instead of bicarbonate) and incubated for 1 hour with 1 uCi (18 uM) (^{14}C)-D,L-3,4-DOPA in 0.5 mL of balanced salt solution to label melanin. The cultures were then washed 3-times in balanced salt solution, and fresh growth medium was added. After 1 day of further incubation in medium to allow efflux of unincorporated label, the cultures were washed 4-times with balanced salt solution, and fresh

medium was added.

On the following day, cultures were washed 1-time with medium and treated with 5 mM 2,4-DOPA. Controls received growth medium alone. After 24 hours of treatment, an aliquot of supernatant was removed and counted in Liquiscint. Monolayers were then washed 3-times with saline and dissolved in 1N KOH overnight. Aliquots of the resultant solution were also counted in Liquiscint. The external standard method was employed to adjust for quenching (see Appendix B). Due to the many washings which the cultures had received during the prelabeling of melanin, partial loss of the monolayer was unavoidable. This was apparent as a bare spot on the plate where the pipet had been located for the washings. To correct for losses which had occurred, data for each individual culture were normalized and expressed as % of total dpm remaining in the monolayer. Total dpm was defined as the dpm present in the monolayer plus the dpm present in the supernatant and was calculated separately for each culture.

Blockade of 2,4-DOPA-Mediated Cytotoxicity by Phenylthiourea (PTU), an Inhibitor of Tyrosinase

$B\text{-}16_2$ cells, grown as replicate monolayer cultures in 2-cm^2 culture wells, were pretreated with either PTU, phenylurea (PU), or thiourea (TU) as indicated in the Table legends. The PU and TU served as controls for PTU. The growth medium was exchanged for medium containing 5 mM 2,4-DOPA with or without PTU, PU, or TU. Control cultures received medium with either PTU, PU, or TU. After 24 hours of treatment, cells were released with trypsin/EDTA and viability

determined by trypan blue exclusion. When studies were carried over for 2 days, spent medium was daily replaced with fresh medium containing 2,4-DOPA with or without PTU, PU, or TU.

Effect of 2,4-DOPA upon DNA, RNA, and Protein Synthesis

The effect of 2,4-DOPA upon macromolecular synthesis was determined by the method of Wick et al. (1979b). B-16 cells and MJY-alpha cells were grown as replicate monolayer cultures in 2-cm^2 culture wells for these experiments. Cells were incubated at 37°C with 0.5 mL of 2,4-DOPA at the concentration and for the time period indicated in the Tables (see Results). Controls received growth medium alone. 2 uCi of either (^{3}H)-thymidine (48 Ci/mmole), (^{3}H)-uridine (60 Ci/mmole), or (^{3}H)-leucine (136 Ci/mmole) in 20 uL of saline was added to the medium already present in the culture well. The cultures were then incubated for an additional hour, washed 2-times with Hank's balanced salt solution, and fixed to the culture well by the addition of 10% TCA. The cultures were then washed 3-times with saline, and dissolved overnight in 1 N KOH. An aliquot was counted in Liquiscint.

Inhibition of Tyrosine Transport by 2,4-DOPA

Transport of (^{3}H)-tyrosine was measured according to the method of Yagi et al (M.J. Yagi, personal communication). $B\text{-}16_2$ cells and MJY-alpha cells, grown in flat-bottom 96-well microtest plates (0.27 cm^2/well), were incubated for 30 minutes in Dulbecco's balanced salt solution containing 6.5 g BSA/L (DBSS/BSA) to deplete intracellular

amino acid pools. Conversion of 2,4-DOPA to 6-hydroxy-DOPA during the experiment was minimized by using 2,4-DOPA at either 25 or 75 uM (K_m = 3.71 mM). The ratio of 2,4-DOPA to tyrosine was adjusted to be similar to that present during cell survival studies by adding 150 nM (^{3}H)-tyrosine (33.4 Curies/mmole). For example, 75 uM 2,4-DOPA produced the same ratio seen with 5 mM 2,4-DOPA in cell survival studies (note: the growth medium used in other experiments contained 10 uM tyrosine). A solution of (^{3}H)-tyrosine was added to control cultures, and a solution of (^{3}H)-tyrosine plus 2,4-DOPA was added to treated cultures. At 30 second intervals, individual wells were washed 3 times with DBSS which was added rapidly to the wells and removed by suction. Monolayer samples were dissolved overnight in 1N KOH and counted.

Incorporation of (^{3}H)-tyrosine into melanin and protein was measured with cells previously incubated for 30 minutes in DBSS/BSA. Melanoma cultures were incubated with (^{3}H)-tyrosine and 2,4-DOPA in DBSS/BSA at room temperature for 9 minutes. Control cultures were incubated with label alone. The cells were then washed 3-times with DBSS which was suctioned off after each wash. After a further 2 minutes incubation, cells were precipitated with 10% TCA, washed 3 times with saline, and dissolved overnight in 200 uL of 1N KOH. The entire KOH solution was counted in 10 mL of Liquiscint.

2.3: MATERIALS

D,L-2,4-DOPA and 2,4-dopamine were gifts from Dr. Albert Manian at the N.I.M.H. The amines, 2,3,4- and 2,4,6-trihydroxy-

phenylethylamine were gifts from Dr. Cyrus Creveling at the N.I.H. Mushroom tyrosinase was obtained from Sigma. Radiochemicals were obtained from either Research Products International [(^{3}H)-thymidine, (^{14}C)-D,L-DOPA, and (^{3}H)-uridine] or New England Nuclear [(^{3}H)-L-leucine and (^{3}H)-L-tyrosine]. All other chemicals were of either reagent, ACS, or HPLC grade.

C57BL/6J mice were obtained from Jackson Labs. The initial B-16 culture, the MJY-alpha mammary tumor culture, and the L-1210 leukemia culture were kindly supplied by Dr. Mary Jane Yagi, in whose lab these cultures are routinely carried. Cloudman melanoma and C-1300 neuroblastoma cultures were obtained from the American Type Culture Collection. The B-16 culture used in later experiments was obtained as tumor tissue growing in C57BL/6J mice and was supplied by Mason Research. Cell culture media, serum, Dulbecco's balanced salt solution, Hank's balanced salt solution, trypsin, trypan blue, and antibiotics were obtained from Gibco. Bovine serum albumin was obtained from Worthington Biochemicals. Culture flasks were either Corning or Falcon brand. 4-Well culture dishes, (2-cm^{2})/well, were obtained from Nunc. Square-bottomed 96-well microtest plates were obtained from Falcon.

2.4: STATISTICAL ANALYSES

Standard statistical tests were employed to evaluate the data. A two-sample t-test was used to compare 2 means. Comparison of the effect of 2,4-DOPA upon the melanized versus non-melanized cell subpopulations within the melanoma cultures was performed with a 1-

tailed paired t-test; the % of control melanized versus non-melanized cells from untreated cultures was compared at each dose of 2,4-DOPA for each day of treatment. When more than one group was compared to a control group, Dunnett's test was used. A 2-tailed test was used to determine whether the group means being compared differed without regard to whether a specific group mean was greater. However, a 1-tailed test was used in order to determine if the group mean being compared differed in a particular direction. Multiple comparisons were made among 3 or more groups by means of the Student-Newman-Keuls test (SNK).

Chapter 3: ENZYMATIC STUDIES: RESULTS AND DISCUSSION

3.1: RESULTS

3.1.1: Experiments with Mushroom Tyrosinase

Characterization of Products

Mushroom tyrosinase, which was readily available, was used in initial experiments to provide a general model for the enzymatic conversion of the prodrugs, 2,4-DOPA and 2,4-dopamine, to 6-hydroxy-DOPA and 6-hydroxydopamine, respectively (see Figure 1-2 for structures). Addition of substrate (i.e., 2,4-DOPA or 2,4-dopamine) to mushroom tyrosinase in the presence of L-3,4-DOPA as cosubstrate resulted in the generation of new products as observed by HPLC (Figure 3-1). The products co-chromatographed with 6-hydroxy-DOPA and 6-hydroxydopamine, respectively. The reaction did not proceed when boiled enzyme was used or when buffer was added in place of active enzyme.

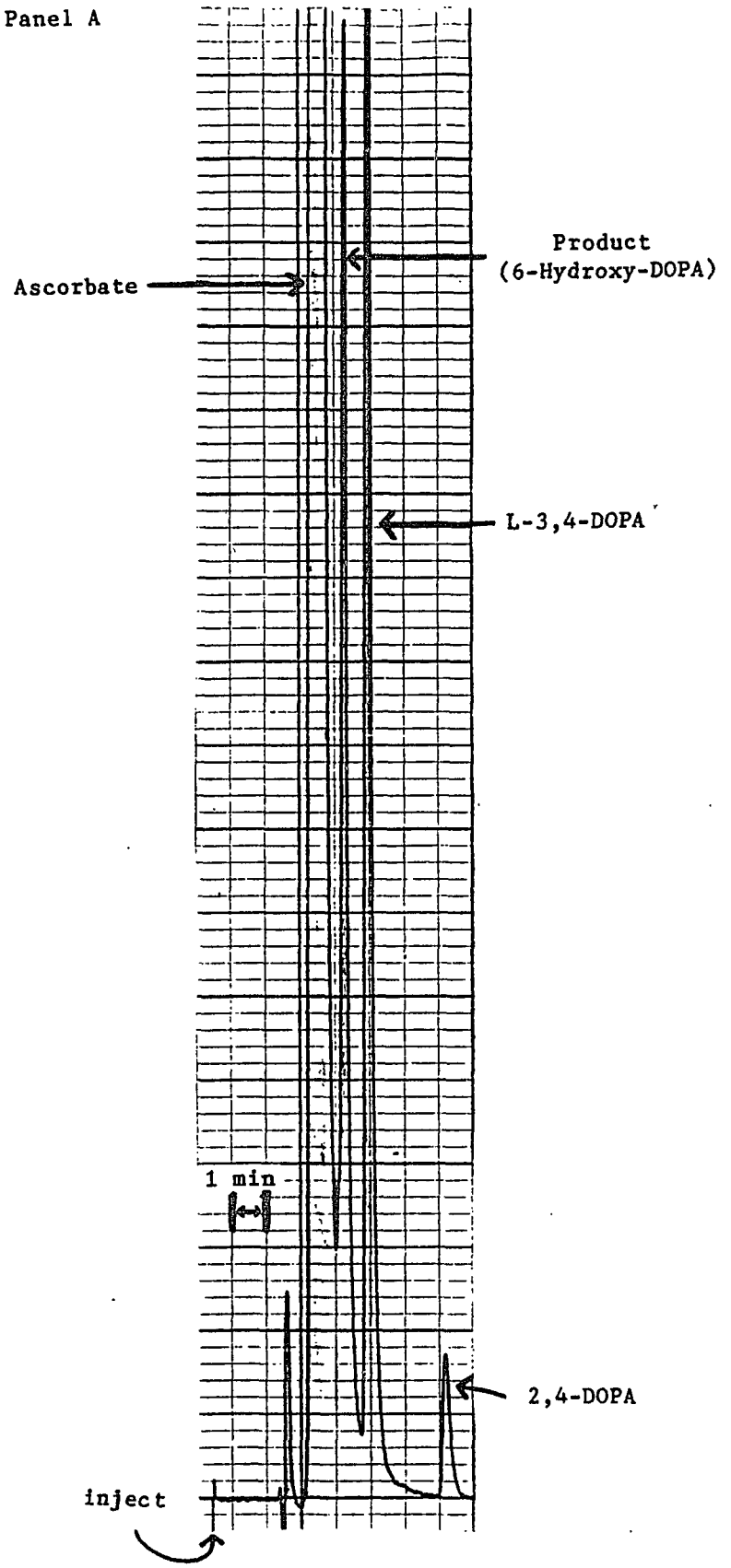
In addition to products generated by hydroxylation in the 5-position, other products might theoretically arise via hydroxylation in the 3- or 6-position to yield the corresponding tri-hydroxy compounds. The possibility existed that alternate products might co-chromatograph with the 5-hydroxylated products; therefore, additional means of identification were needed.

Standards of 2,3,4- and 2,4,6-trihydroxyphenylethylamine were available for comparison with 2,4,5-trihydroxyphenylethylamine (i.e.,

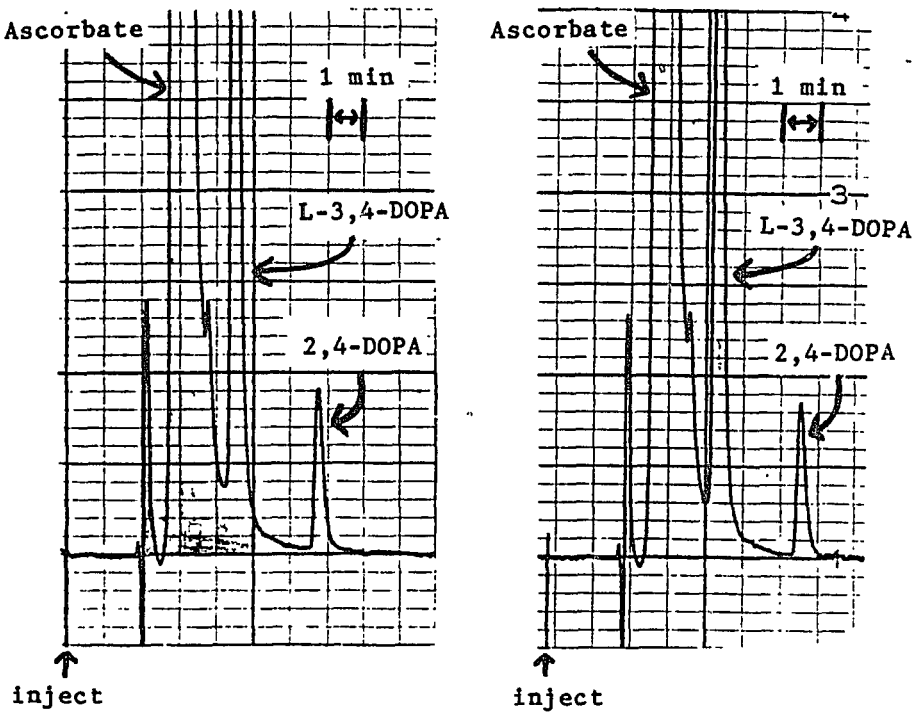
Figure 3-1. HPLC Chromatograms Demonstrating the Synthesis of 6-Hydroxy-DOPA (A-C) and 6-Hydroxydopamine (D-F) by Mushroom Tyrosinase

Panels A-C. Mushroom tyrosinase (3.500×10^{-3} units) was added to a solution of 5 mM 2,4-DOPA (substrate), 50 uM L-3,4-DOPA (cosubstrate), and 1 mM ascorbate in 50 mM phosphate buffer, pH 6.8, containing 100 uM DTPA in a final volume of 4.2 mLs. An aliquot was removed after 6 minutes, acidified, centrifuged, and analyzed by HPLC with electrochemical detection (electrode potential = +0.8 volts vs. a Ag/AgCl reference). 6-Hydroxy-DOPA (product) was separated from starting materials by a mobile phase of 50 mM phosphate buffer, pH 3.0, containing 1 mM EDTA, at a flow rate of 1.5 mLs/minute. An Altex 5 um C-18 column was used. Panel A demonstrates the enzymatic synthesis of 6-hydroxy-DOPA (present at 14.95 uM in acidified sample). Ascorbate elutes in the void volume, before 6-hydroxy-DOPA, while L-3,4-DOPA and 2,4-DOPA elute after 6-hydroxy-DOPA. (At a potential of +0.8 volts, 5 mM 2,4-DOPA does not give rise to a large peak). Panel B shows a chromatogram of a control sample to which boiled enzyme was added instead of active enzyme. A small, constant, peak can be seen immediately before the position at which 6-hydroxy-DOPA would elute. In both panels A and B, 20 uLs of sample were injected. Panel C demonstrates that the enzymatic product co-chromatographs with a standard of 6-hydroxy-DOPA. Enzymatic product (74.8 pmoles) and a standard of 6-hydroxy-DOPA (117.0 pmoles) were loaded into the same syringe and injected. The mixture gave rise to a single, smooth peak, thereby satisfying the criterion of co-chromatography.

Panels D-F. Mushroom tyrosinase (2.352×10^{-3} units) was added to a solution of 5 mM 2,4-dopamine (substrate), 50 uM L-3,4-DOPA (cosubstrate), and 1 mM ascorbate in 50 mM phosphate buffer, as above, sampled at 6 minutes, acidified, centrifuged, and analyzed by HPLC with electrochemical detection (electrode potential = +0.9 volts versus a Ag/AgCl reference). 6-Hydroxydopamine (product) was separated from starting materials by a mobile phase of 50 mM acetate buffer, pH 5.1, containing 1 mM EDTA, 2.5 mM sodium octyl sulfate, and 20% methanol, at a flow rate of 2 mLs/minute. A Waters 10 um C-18 column was used. Panel D demonstrates the enzymatic synthesis of 6-hydroxydopamine (16.00 uM in acidified sample). Ascorbate and L-3,4-DOPA elute in the void volume, before 6-hydroxydopamine, while 2,4-dopamine elutes after 6-hydroxydopamine. Panel E shows chromatograms of control samples. The top of the panel (same conditions as Panel D) shows chromatograms of samples to which buffer was substituted for active enzyme. In the bottom of the panel (left and middle, same conditions as Figure 3-3) boiled enzyme was substituted. (The bottom right shows a chromatogram of product under these conditions). Panel F demonstrates that the tyrosinase-generated product co-chromatographs with a standard of 6-hydroxydopamine. Tyrosinase-generated product (120.0 pmoles) and a standard of 6-hydroxydopamine (119.5 pmoles) were loaded into the same syringe and injected. The mixture gave rise to a single, smooth peak, thereby satisfying the criterion of co-chromatography.


Panel A
Product
(6-Hydroxy-DOPA)
Ascorbate
L-3,4-DOPA
1 min
2,4-DOPA
inject

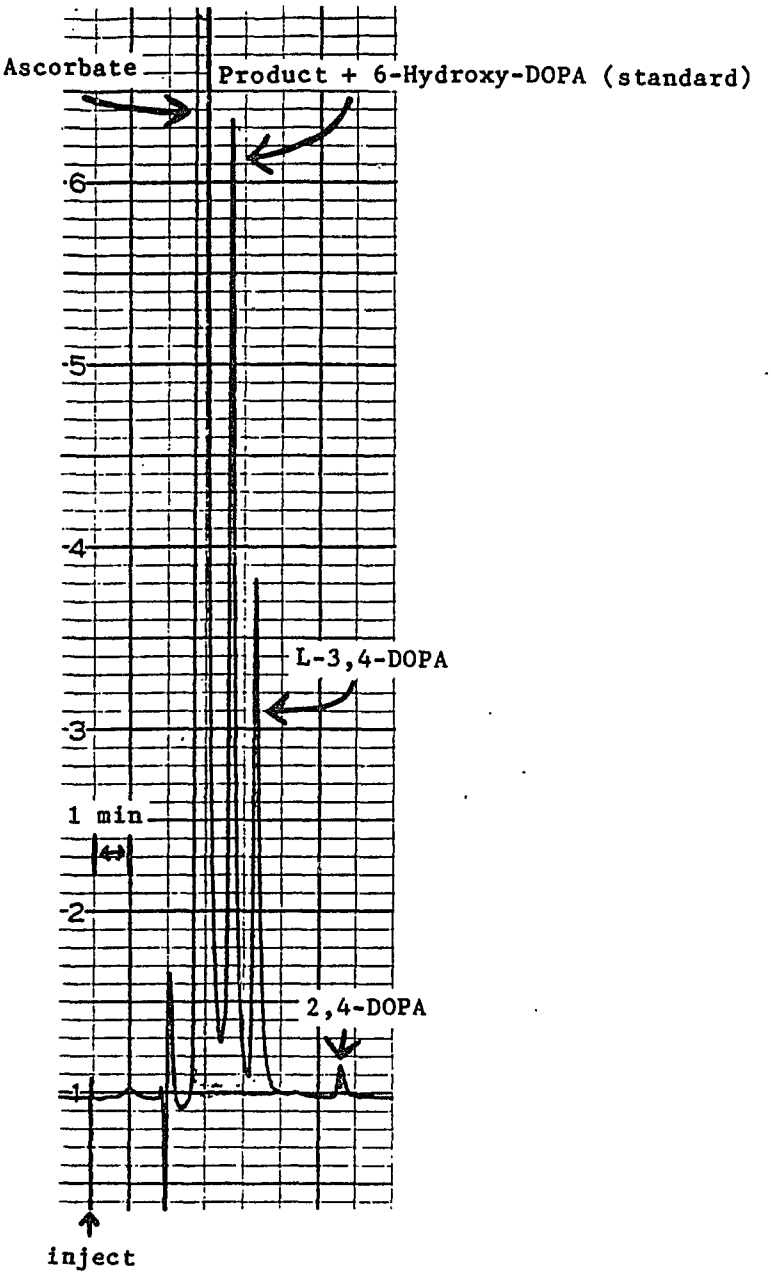
Panel B



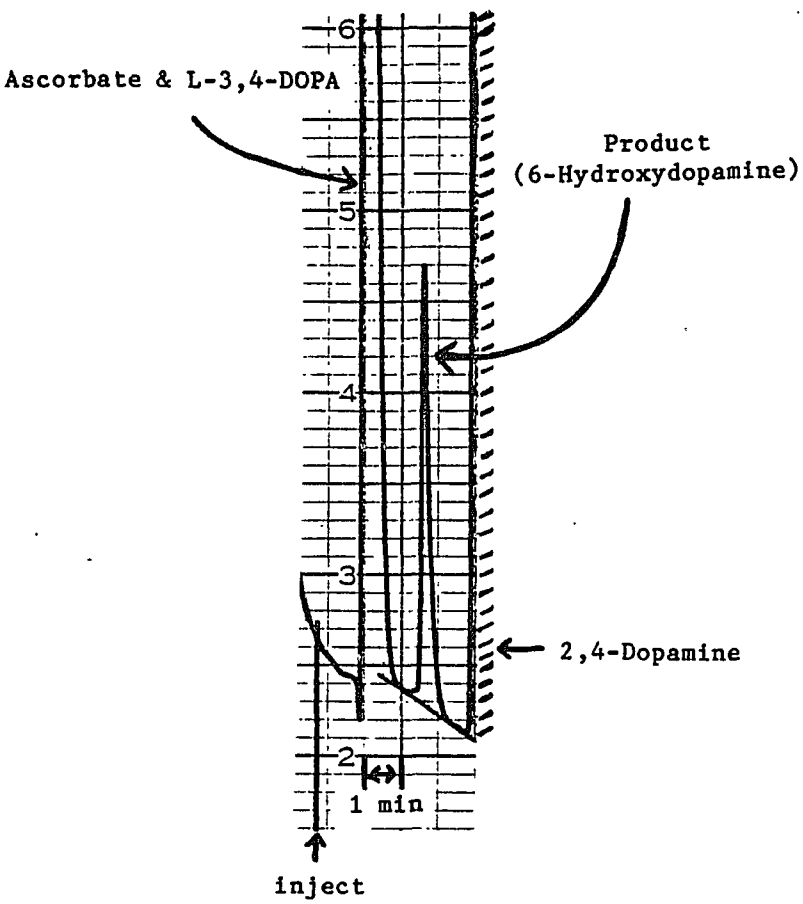
Control (0 min)

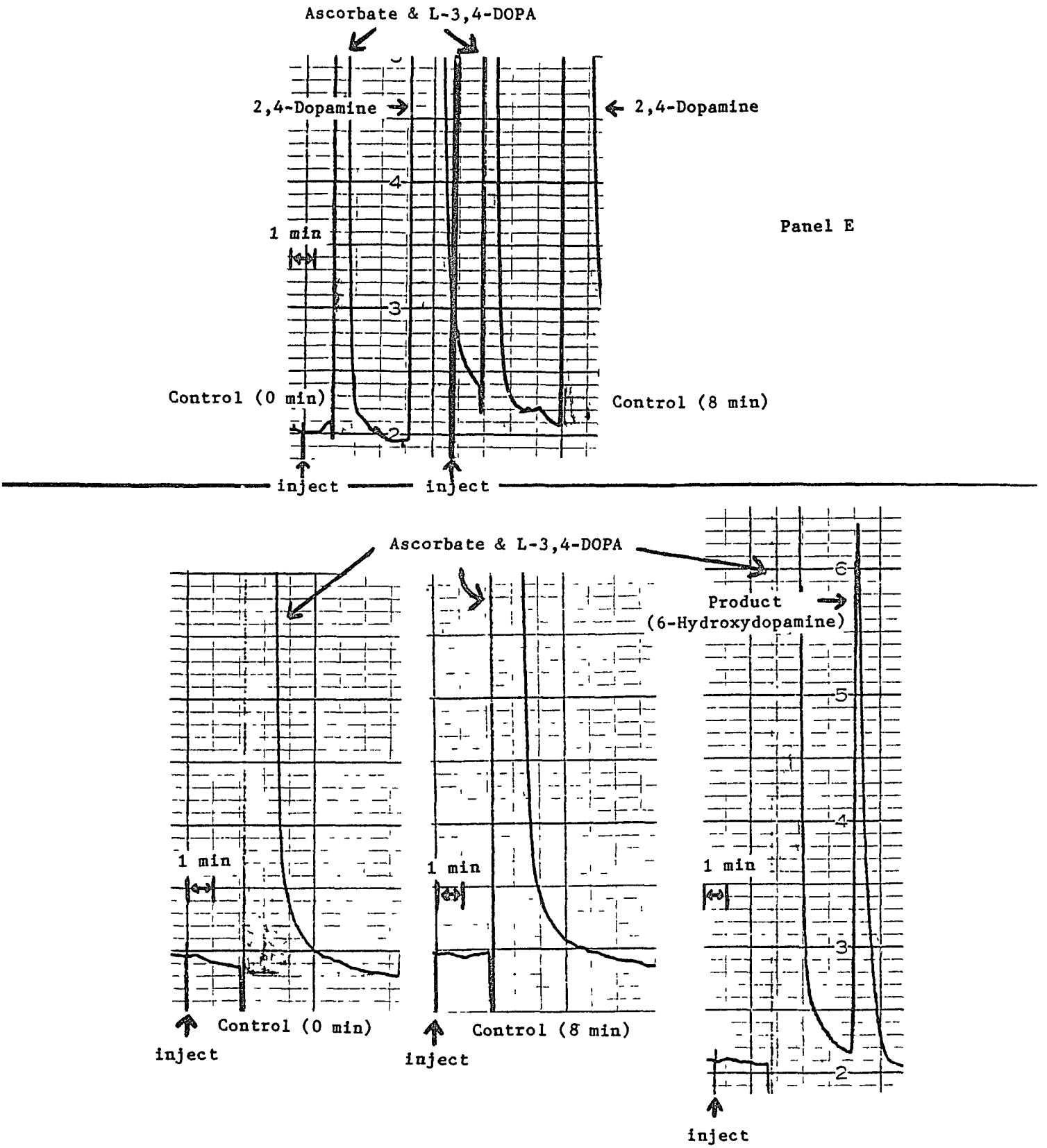
Control (8 min)

Panel C

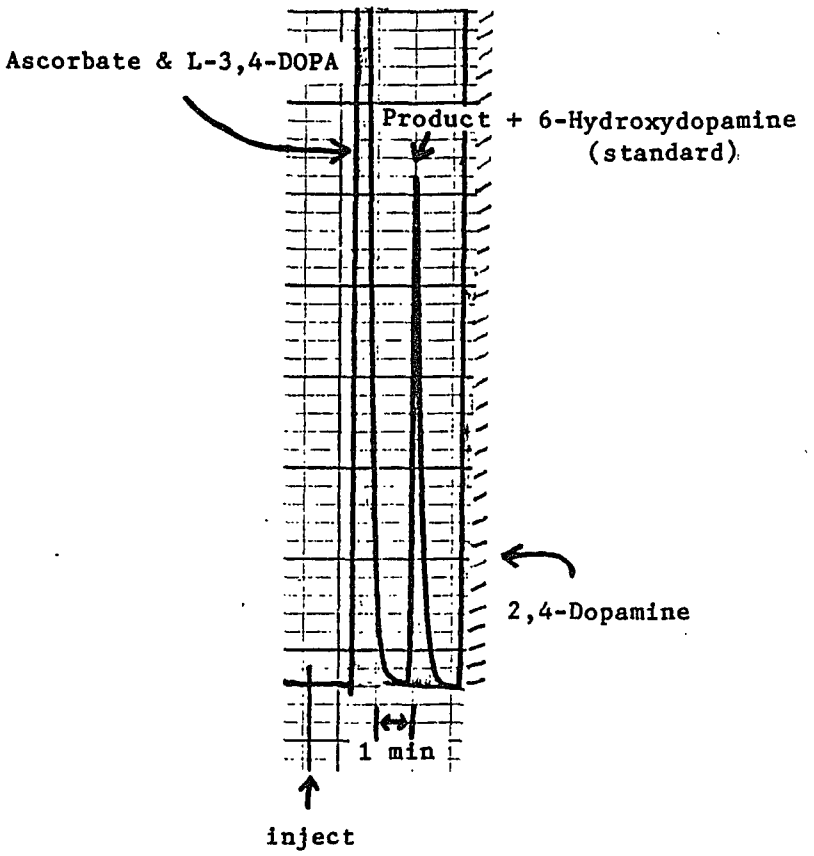

Ascorbate
Product + 6-Hydroxy-DOPA (standard)
6
5
4
L-3,4-DOPA
3
1 min
2
2,4-DOPA
1
inject

Panel D


Ascorbate & L-3,4-DOPA
Product
(6-Hydroxydopamine)
6
5
4
3
2
2,4-Dopamine
1 min
inject


Ascorbate & L-3,4-DOPA
2,4-Dopamine
2,4-Dopamine
1 min
Control (0 min)
Control (8 min)
inject
inject
Panel E
Ascorbate & L-3,4-DOPA
Product
(6-Hydroxydopamine)
1 min
1 min
1 min
Control (0 min)
Control (8 min)
inject
inject
inject

Panel F


Ascorbate & L-3,4-DOPA
Product + 6-Hydroxydopamine
(standard)
2,4-Dopamine
1 min
inject

6-hydroxydopamine). These compounds could be separated from 6-hydroxydopamine by HPLC (Figure 3-2) and were not detected in samples of enzyme-generated 6-hydroxydopamine (Figure 3-3). Therefore, hydroxylation of 2,4-dopamine occurs predominantly in the 5-position.

The enzyme-generated products were further characterized (Figure 3-4) by their electrochemical properties (Morrison and Cohen 1983) at the pH of the HPLC mobile phase. The half-wave potential of enzyme-generated 6-hydroxy-DOPA at pH 3.0 was graphically determined to be +0.39 volts versus a Ag/AgCl reference electrode. This was similar to that of a standard of 6-hydroxy-DOPA (+0.38 volts). For comparison, a standard of L-3,4-DOPA was found to have a half-wave potential of +0.64 volts. The half-wave potential at pH 5.0 of enzyme-generated 6-hydroxydopamine was +0.15 volts, identical to that of the standard. 3,4-DA was found to have a half-wave potential of +0.43 volts. The data show that the enzyme-generated products have half-wave potentials corresponding to 6-hydroxy-DOPA and 6-hydroxydopamine.

Characteristics of the Reaction of 2,4-DOPA and 2,4-Dopamine with Mushroom Tyrosinase

The generation of 6-hydroxy-DOPA and 6-hydroxydopamine from 2,4-DOPA and 2,4-dopamine, respectively, were blocked (Table 3-1) by phenylthiourea (PTU), an inhibitor of tyrosinase (Lerner and Fitzpatrick 1950), thereby demonstrating the enzymatic nature of the reactions. 100 uM PTU inhibited the reaction >95% when either substrate was present at 5 mM. L-3,4-DOPA (cosubstrate) was present at 50 uM and ascorbate, 1 mM. The dose-response relationship was

Figure 3-2. HPLC Separation of 2,3,4-, 2,4,6-, and 2,4,5-Trihydroxyphenylethylamine (6-Hydroxydopamine)

Standards were separated by a mobile phase of 50 mM acetate buffer, pH 5.1, containing 1 mM EDTA, 2.5 mM sodium octyl sulfate, and 20% methanol, at a flow rate of 1 mL/minute. A Waters 10 um C-18 column was used. The detector electrode was set at +0.9 volts vs. a Ag/AgCl reference electrode. The order of elution was 2,4,5-trihydroxyphenylethylamine first, followed by 2,3,4-, then 2,4,6-trihydroxyphenylethylamine.

Trihydroxyphenylethylamines

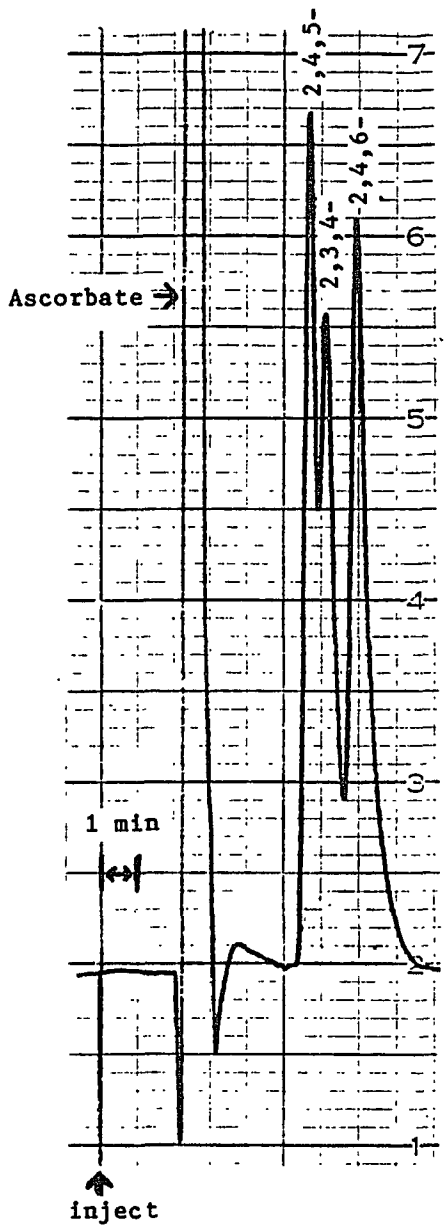


Figure 3-3. HPLC Chromatogram Demonstrating the Absence of 2,3,4- and 2,4,6-Trihydroxyphenylethylamine in Samples of Mushroom Tyrosinase-Generated 6-Hydroxydopamine

Mushroom tyrosinase (2.352×10^{-3} units) was added to a final volume of 4.2 mLs of 50 mM phosphate buffer, pH 6.8, containing 1 mM 2,4-dopamine (substrate), 50 uM L-3,4-DOPA (cosubstrate), 1 mM ascorbate, and 100 uM DTPA. After 6 minutes, an aliquot was removed, acidified, and analyzed by HPLC with electrochemical detection (electrode potential +0.9 volts vs. a Ag/AgCl reference). 6-Hydroxydopamine (product) was separated from starting materials by a mobile phase of 50 mM acetate buffer, pH 5.1, containing 1 mM EDTA, 2.5 mM sodium octyl sulfate, and 20% methanol. A chromatogram of tyrosinase-generated 6-hydroxydopamine (196 pmoles injected) is shown. No peaks corresponding to 2,3,4- or 2,4,6-trihydroxyphenylethylamine are evident.

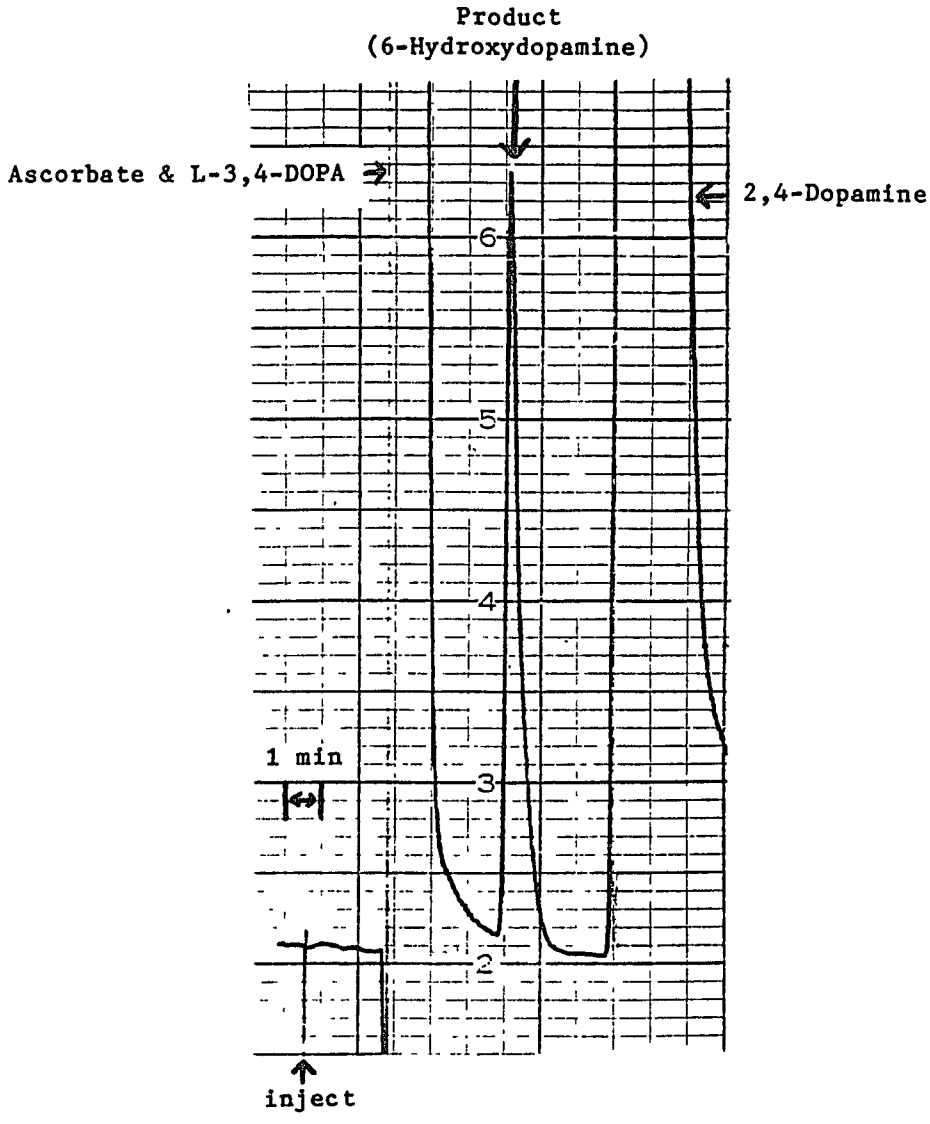
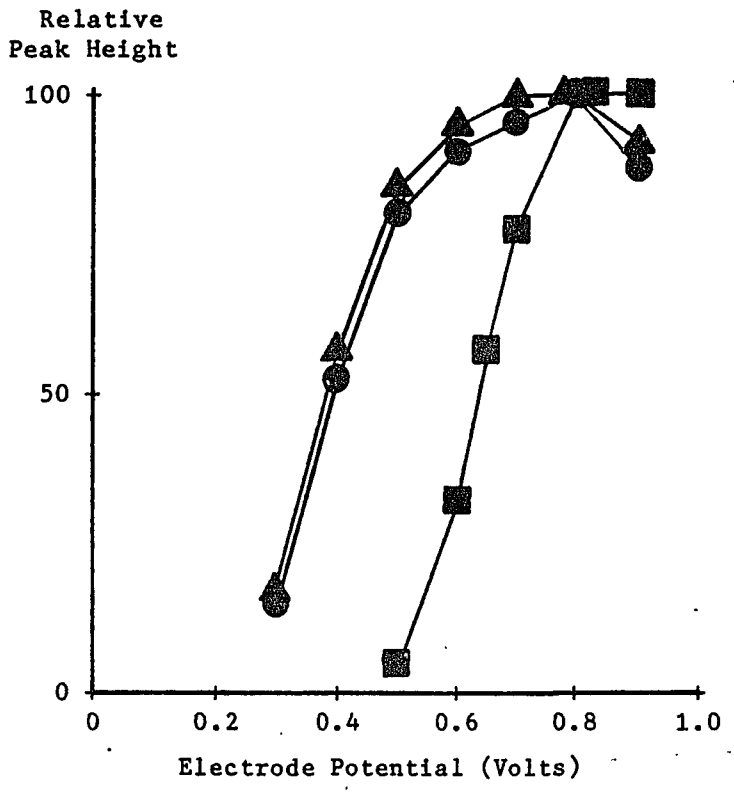

Product
(6-Hydroxydopamine)
Ascorbate & L-3,4-DOPA
2,4-Dopamine
6
5
4
3
2
1 min
inject

Figure 3-4. Electrochemical Oxidation Properties of Standards and Mushroom Tyrosinase-Generated Products

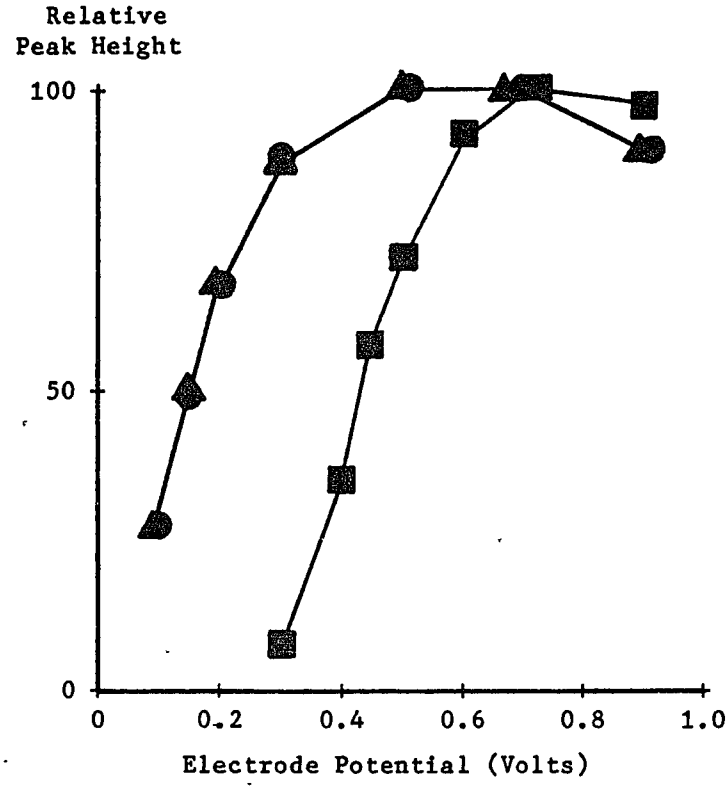
Products and standards were analyzed by HPLC with electrochemical detection. Variation of the electrode potential between replicate injections allowed determination of the electrochemical oxidation properties of the products and standards at the pH of the mobile phase.

Panel A shows the relative peak heights for mushroom tyrosinase-generated 6-hydroxy-DOPA (circles, 100 = 23.4 nAmps), authentic 6-hydroxy-DOPA (triangles, 100 = 15.7 nAmps), and L-3,4-DOPA (squares, 100 = 16.0 nAmps). A mobile phase consisting of 50 mM phosphate buffer, pH 3.0, containing 1 mM EDTA, was used at a flow rate of 1.5 mL/minute. The average standard deviation for duplicate determinations was 1.1 relative peak height units (range: 0.0 to 3.6).

Panel B shows the relative peak heights for mushroom tyrosinase-generated 6-hydroxydopamine (circles, 100 = 20.6 nAmps), authentic 6-hydroxydopamine (triangles, 100 = 23.4 nAmps), and 3,4-dopamine (squares, 100 = 28.4 nAmps). A mobile phase consisting of 50 mM acetate buffer, pH 5.1, containing 1 mM EDTA, 2.5 mM sodium octyl sulfate, and 20% methanol, was used at a flow rate of 2 mL/minute. The average standard deviation for duplicate determinations was 0.9 relative peak height units (range: 0.0 to 2.4).


Relative
Peak Height
100
50
0
0
0.2
0.4
0.6
0.8
1.0
Electrode Potential (Volts)

Panel A


Relative
Peak Height
100
50
0
0
0.2
0.4
0.6
0.8
1.0
Electrode Potential (Volts)

Panel B

Table 3-1

Phenylthiourea (PTU), an Inhibitor of Tyrosinase, Blocks Conversion of Prodrugs by Tyrosinase

Prodrug (5 mM)	PTU (uM)	Rate of Product Formation (S.D.) umole/min/unit	% Inhibition
a 2,4-DOPA	0	1.80 (0.11)	0.0
	1	0.78 (0.36)	56.5
	10	0.03 (0.05)	98.2
	100	0.04 (0.04)	97.9
b 2,4-DA	0	4.19 (0.36)	0.0
	100	0.17 (0.06)	95.9
c 2,4-DOPA	0	2.69 (0.32)	0.0
	100	0.18 (0.18)	93.3

The formation of product was monitored by HPLC. The rate of product formation was calculated from a least-squares fit of data at either 2,4,and 6 minutes (mushroom tyrosinase) or 2,6, and 10 minutes (B-16 tyrosinase).

[a]Mushroom tyrosinase (1.66×10^{-3} units) was added to substrate in the presence of 50 uM L-3,4-DOPA, 1 mM ascorbate, 100 uM DTPA, and PTU in 50 mM phosphate buffer, pH 6.8, 22°C.

[b]Mushroom tyrosinase (2.63×10^{-3} units) was added to initiate the reaction. Other components were present as in "a."

[c]B-16 tyrosinase (4.46×10^{-4} units of the 40-60% ammonium sulfate fraction) was added to initiate the reaction. Ascorbate was present at 0.1 mM and sodium cholate was present at 0.1%. All other components were present as in "a."

examined with 5 mM 2,4-DOPA as substrate. It was found that as little as 1 uM PTU inhibited the reaction by 56.5%.

Representative plots of product accumulation with time are shown in Figure 3-5. The reactions were linear with time, although extrapolation back to time zero revealed that the lines intersected the y-axis. The linear portion of the curve was defined as the "steady-state", whereas the y-intercept represented a "burst" in product formation.

The burst increased when either substrate or L-3,4-DOPA concentration was increased (Figure 3-6, panels A and B). As discussed in the next section, L-3,4-DOPA serves as a cosubstrate for tyrosinase. The dependence of the burst upon substrate and cosubstrate concentrations illustrates the enzymatic nature of the burst. In addition, the burst was either greatly decreased or completely abolished by PTU (tyrosinase inhibitor) when 2,4-DOPA or 2,4-dopamine were used as substrates, respectively (Table 3-2). This observation confirms the enzymatic nature of the burst.

The metal-chelator, DTPA, was present in the reaction mixture to suppress metal-catalyzed autoxidation of product and ascorbate. Experiments were run to determine whether DTPA had any effect upon the enzymatic activity that might explain the burst (i.e., if DTPA could remove copper from the active site, then the rate might decrease with time. Note: DTPA was not present in the enzyme stock solution). When 0.0063 mg mushroom tyrosinase/mL was added to 2.5 mM L-3,4-DOPA in 50 mM phosphate buffer, pH 6.8, the absorbance increased by 0.147/min ($\pm$ 0.003, S.D., N = 3). After preincubation of the

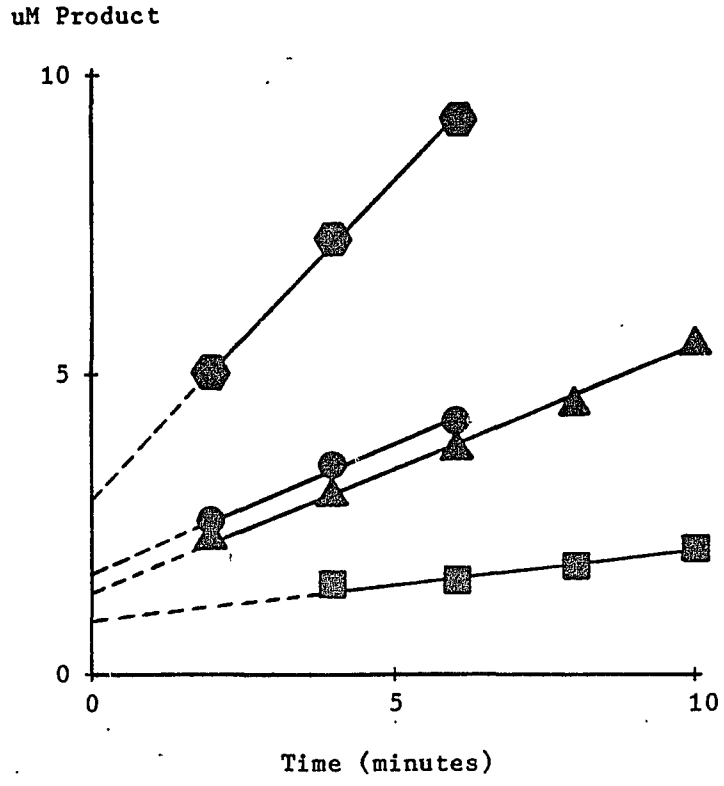


Figure 3-5. Generation of 6-Hydroxy-DOPA and 6-Hydroxydopamine from 2,4-DOPA and 2,4-Dopamine by Mushroom Tyrosinase

Substrate was incubated with mushroom tyrosinase in the presence of L-3,4-DOPA (cosubstrate) and 1 mM ascorbate (maintains the product in the reduced state) in 4.2 mLs of 50 mM phosphate buffer, pH 6.8, containing 100 uM DTPA. Timed aliquots were acidified, centrifuged, and analyzed for product by HPLC with electrochemical detection. The data were obtained from the experiments shown in Figure 3-8. In experiments with 2,4-dopamine, 2.352×10^{-3} units of mushroom tyrosinase were added to each flask. In experiments with 2,4-DOPA, 1.764×10^{-3} units of enzyme were added.

Hexagon = 1 mM 2,4-Dopamine + 10 uM L-3,4-DOPA

Circle = 1 mM 2,4-Dopamine + 1 uM L-3,4-DOPA

Triangle = 1 mM 2,4-DOPA + 10 uM L-3,4-DOPA

Square = 1 mM 2,4-DOPA + 1 uM L-3,4-DOPA

Figure 3-6. Mushroom Tyrosinase Generates an Initial Burst of Product from Either 2,4-DOPA or 2,4-Dopamine

The reactions of mushroom tyrosinase were linear with time; however, extrapolation of the data back to time zero revealed that the lines intersected the y-axis (see Figure 3-5 for representative plots). The y-intercept, designated as the "burst," represented the amount of product rapidly formed before the reaction proceeded at the slower steady-state rate. The data were obtained from the experiments shown in Figures 3-7 to 3-9. The reactions were performed in 50 mM phosphate buffer, pH 6.8, containing 1 mM ascorbate and 100 uM DTPA.

Panel A shows the variation of the burst with substrate concentration:

Circle = 2,4-Dopamine + 50 uM L-3,4-DOPA (N=2)

Square = 2,4-DOPA + 50 uM L-3,4-DOPA (N=4)

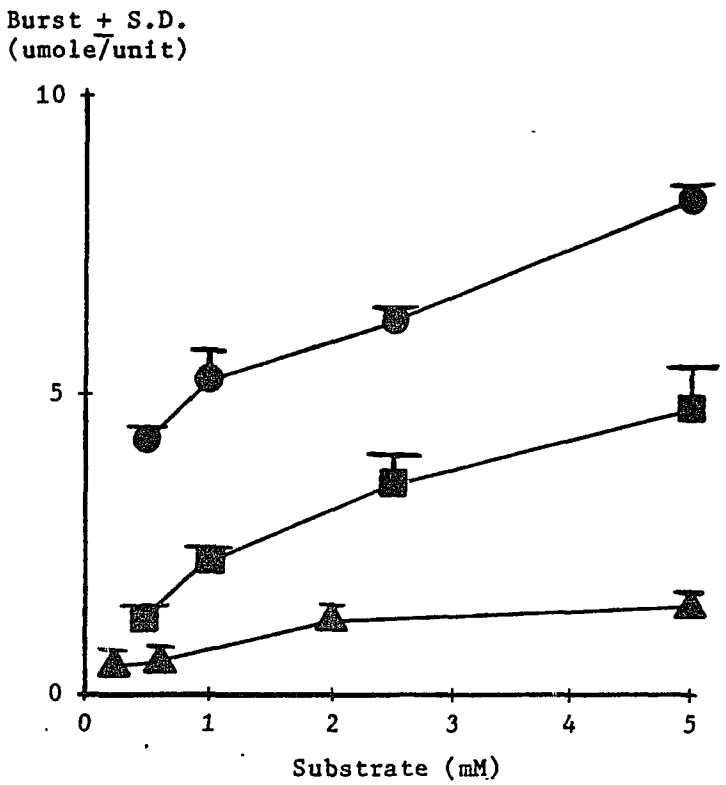
Triangle = 2,4-DOPA + 5 uM L-3,4-DOPA (N=2)

Panel B shows the variation of the burst with L-3,4-DOPA concentration:

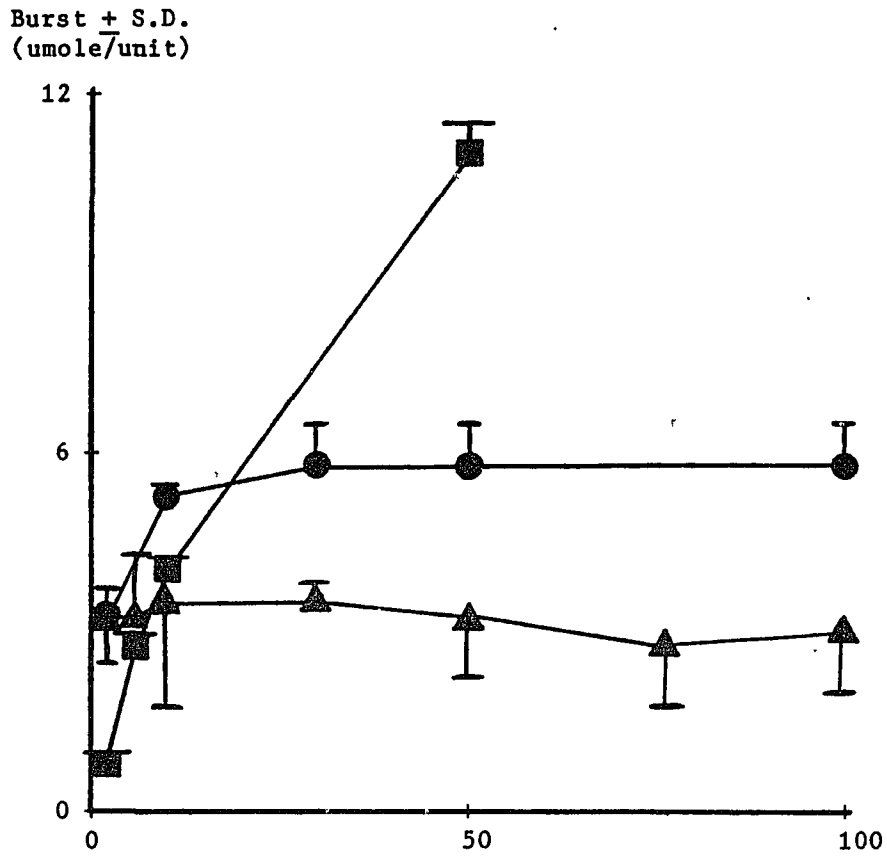
Circle = 1 mM 2,4-Dopamine (N=2, except for 50 uM L-3,4-DOPA where N=3)

Square = 5 mM 2,4-DOPA (N=2)

Triangle = 1 mM 2,4-DOPA (N=3, except for 1 uM L-3,4-DOPA where N=4)



Panel A



Panel B

Table 3-2

Phenylthiourea (PTU), an Inhibitor of Tyrosinase, Blocks the Burst Effect

Prodrug (5 mM)	PTU (mM)	Burst (S.D.) (umole/min)	% Inhibition
2,4-DOPA	0	5.94 (0.41)	0.0
	1	1.49 (0.44)	74.9
	10	1.90 (0.26)	68.0
	100	0.84 (0.26)	85.9
2,4-DA	0	8.65 (1.12)	0.0
	100	-0.05 (0.02)	100.6

The data are from the experiments shown in Table 3-1 which utilized mushroom tyrosinase. The burst was determined by extrapolation of data points to zero time. A positive burst represents the amount of product rapidly formed before steady-state is acheived. A negative burst represents a "lag" before the reaction proceeds at the steady-state rate.

tyrosinase stock for 10 minutes in buffer containing 100 uM DTPA, the absorbance increased by 0.148/min (± 0.002 S.D., N = 3) in assay solution containing 100 uM DTPA. Therefore, it was concluded that DTPA did not affect tyrosinase activity, at least over the period of time used in the reported experiments.

Kinetic Parameters and pH Dependence of the Reactions of 2,4-DOPA and 2,4-Dopamine with Mushroom Tyrosinase

Experiments were performed in which the concentration of either substrate or cosubstrate (L-3,4-DOPA) was varied in order to determine kinetic parameters. When substrate concentration was varied while holding L-3,4-DOPA concentration constant at 50 uM, the resultant double-reciprocal plots were linear (Figure 3-7) yielding apparent K_m values of 1.25 mM and 1.26 mM for 2,4-DOPA and 2,4-dopamine, respectively. The apparent V_{max} was 2.39 umole/min/unit with 2,4-DOPA as substrate and 5.11 umole/min/unit with 2,4-dopamine as substrate (Table 3-3).

When the concentration of L-3,4-DOPA was varied while holding the concentration of substrate constant at 1 mM, a stimulatory effect of L-3,4-DOPA upon the rate was evident (Figure 3-8). Regression of 1/V versus 1/[L-3,4-DOPA] yielded an apparent $K_{activator}$ of 2.5 uM when 1 mM 2,4-DOPA was used as substrate and 1.3 uM when 5 mM 2,4-DOPA was used as substrate. In the presence of 1 mM 2,4-dopamine, the apparent $K_{activator}$ was 1.9 uM (Table 3-3). These data indicate that the apparent $K_{activator}$ for L-3,4-DOPA with mushroom tyrosinase is relatively constant.

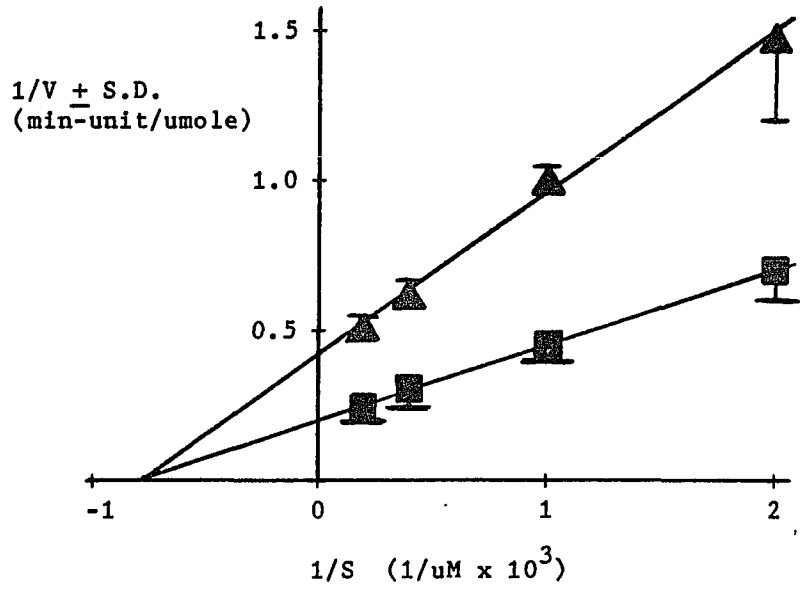


Figure 3-7. Conversion of 2,4-DOPA and 2,4-Dopamine to 6-Hydroxy-DOPA and 6-Hydroxydopamine, Respectively, by Mushroom Tyrosinase in the Presence of 50 uM L-3,4-DOPA; Double-Reciprocal Analysis

Mushroom tyrosinase (2.352×10^{-3} units) was added to a final volume of 4.2 mLs of 50 mM phosphate buffer, pH 6.8, containing 50 uM L-3,4-DOPA (cosubstrate), 1 mM ascorbate, 100 uM DTPA, and either 2,4-DOPA (triangles, N=4) or 2,4-dopamine (squares, N=2). Timed aliquots were acidified, centrifuged, and analyzed for product by HPLC with electrochemical detection.

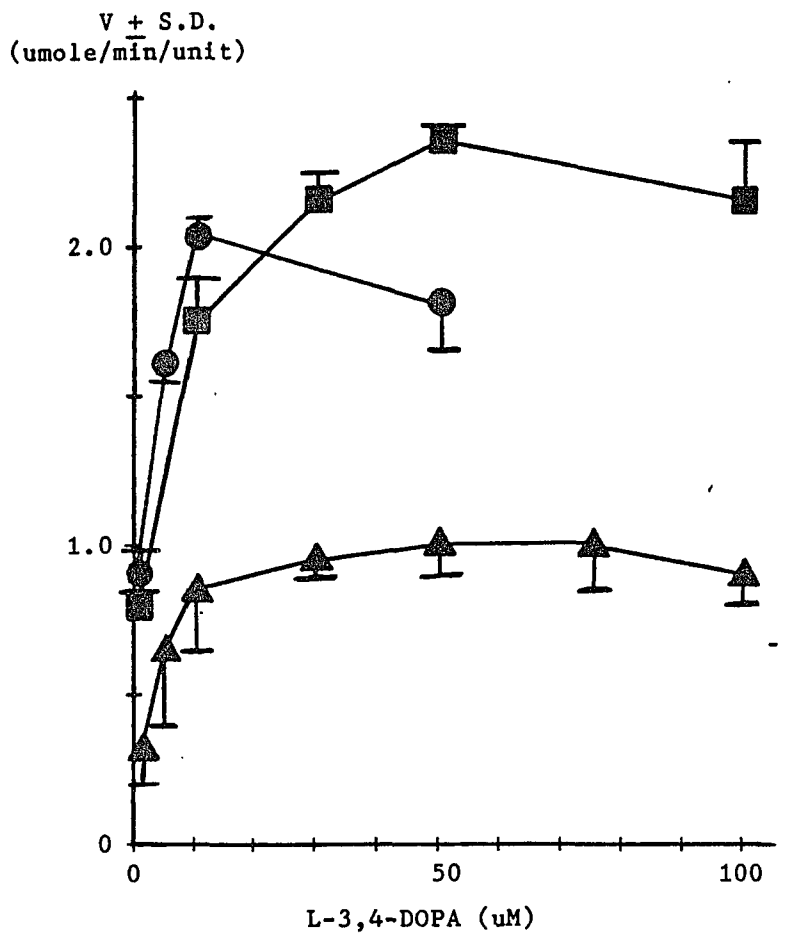


Figure 3-8. Characterization of L-3,4-DOPA as Cosubstrate for Mushroom Tyrosinase-Catalyzed Hydroxylations

Mushroom tyrosinase was added to a final volume of 4.2 mLs of 50 mM phosphate buffer, pH 6.8, containing 1 mM ascorbate, 100 uM DTPA, and either 1 mM 2,4-dopamine (squares, N=2), or 1 mM 2,4-DOPA (triangles, N=3, except for 1 uM L-3,4-DOPA where N=4), or 5 mM 2,4-DOPA (circles, N=2). Mushroom tyrosinase was present at either 2.352 x 10^{-3} units (squares), or 1.764 x 10^{-3} units (triangles), or 1.512 x 10^{-3} units (circles). Timed aliquots were acidified, centrifuged, and analyzed by HPLC with electrochemical detection.

Table 3-3

Summary of Kinetic Parameters for B-16 and Mushroom Tyrosinases

	Parameter ± S.E. (N)		
	B-16 Tyrosinase	Mushroom Tyrosinase	
	2,4-DOPA	2,4-DOPA	2,4-Dopamine
Km (50 uM L-3,4-DOPA)	3.71 mM ± 1.36 (9)	1.25 mM ± 0.18 (16)	1.26 mM ± 0.18 (8)
Vmax (50 uM L-3,4-DOPA)	4.43 umole/min/unit ± 1.59 (9)	2.39 umole/min/unit ± 0.29 (16)	5.11 umole/min/unit ± 0.59 (8)
K(activator) (1 mM substrate)	3.1 uM ± 0.4 (15)	2.5 uM ± 0.4 (22)	1.9 uM ± 0.1 (11)
K(activator) (5 mM substrate)		1.3 uM ± 0.1 (8)	
Km (5 uM L-3,4-DOPA)		0.43 mM ± 0.04 (8)	
Vmax (5 uM L-3,4-DOPA)		1.10 umole/min/unit ± 0.09 (8)	
pH Maximum	8.0	9.0	5.5 - 7.5

Product formation was monitored by HPLC. pH maxima were determined by inspection of data. Other kinetic parameters were determined by double-reciprocal analysis. Where substrate concentration was varied, the concentration of L-3,4-DOPA (cosubstrate) is specified in parentheses. Where L-3,4-DOPA concentration was varied, the substrate concentration is specified in parentheses.

To determine whether the activating effect of L-3,4-DOPA was brought about by a change in K_m, V_{max}, or both, 2,4-DOPA concentration was varied while holding the concentration of L-3,4-DOPA constant at 5 uM (Figure 3-9). Under these conditions, the apparent K_m was 0.43 mM, and the V_{max} was 1.10 umole/min/unit (Table 3-3); (cf. the values of K_m = 1.25 mM and V_{max} = 2.39 umole/min/unit when L-3,4-DOPA was held constant at 50 uM; see above). Therefore, it appears that both the apparent K_m and V_{max} vary directly with the concentration of L-3,4-DOPA.

The pH dependence of the reactions was determined using 1 mM substrate with 30 uM L-3,4-DOPA as cosubstrate (Figure 3-10). The pH maximum with 2,4-DOPA as substrate was approximately 9.0. When 2,4-dopamine was used as substrate, there was a broad pH maximum over the range of 5.5 to 7.5.

3.1.2: Experiments with B-16 Melanoma Tyrosinase

Preparation of B-16 Tyrosinase

Kinetic experiments were performed with enzyme isolated in a 40-60% ammonium sulfate fraction of B-16 tumor tissue; the tumor was grown subcutaneously in C57BL/6J mice. Table 3-4 outlines the partial purification. The crude homogenate contained 0.18 units/gm tissue when assayed at 25°C. by the dopachrome method (Pomerantz 1963). Most of the activity was recovered in the 40-60% ammonium sulfate fraction. In comparison, a crude homogenate of human melanoma tissue, assayed at 37°C. by the same method, contained 0.14 units/gm tissue (Nishioka

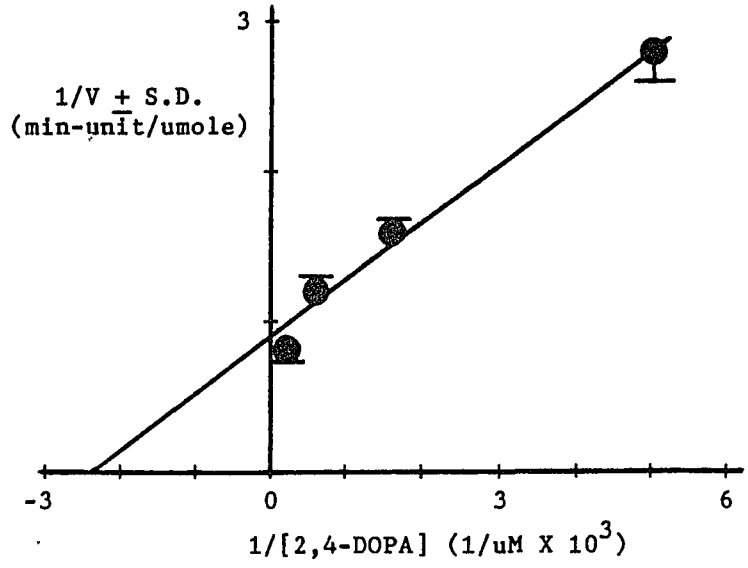


Figure 3-9. Conversion of 2,4-DOPA to 6-Hydroxy-DOPA by Mushroom Tyrosinase in the Presence of 5 uM L-3,4-DOPA; Double-Reciprocal Analysis

Mushroom tyrosinase (5.221×10^{-3} units) was added to a final volume of 4.2 mLs of 50 mM phosphate buffer, pH 6.8, containing 5 uM L-3,4-DOPA, 1 mM ascorbate, 100 uM DTPA, and 2,4-DOPA. Timed aliquots were acidified, centrifuged, and analyzed by HPLC with electrochemical detection. Each point represents the mean of 2 experiments.

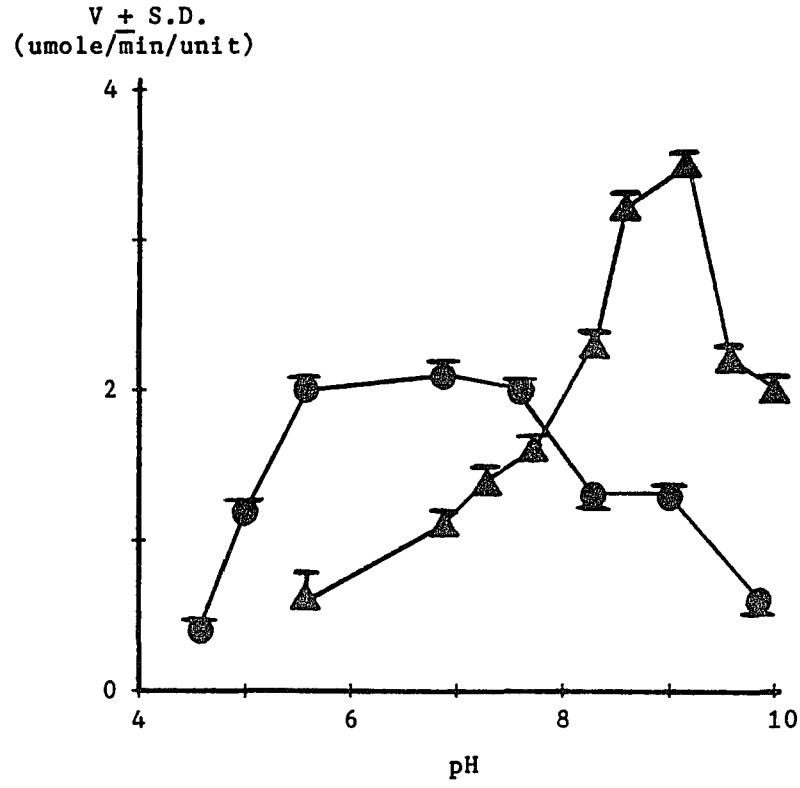


Figure 3-10. pH Dependence of the Conversion of 2,4-DOPA to 6-Hydroxy-DOPA and 2,4-Dopamine to 6-Hydroxydopamine by Mushroom Tyrosinase

Mushroom tyrosinase was added to a final volume of 4.2 mLs of 50 mM buffer (see methods) containing 1 mM ascorbate, 100 uM DTPA, 30 uM L-3,4-DOPA (cosubstrate), and either 1 mM 2,4-DOPA (triangles, N=2, except at pH 6.8 where N=3) or 1 mM 2,4-dopamine (circles, N=2). Mushroom tyrosinase was present at either 1.764×10^{-3} units (triangles) or 2.352×10^{-3} units (circles). Timed aliquots were acidified, centrifuged, and analyzed by HPLC with electrochemical detection.

Table 3-4

Preparation of B-16 Tyrosinase

Fraction	Dopachrome Units[a] (S.D.), N=2	% Recovery
Homogenate Supernatant[b]	1.23 (0.05)	100
40-60% Ammonium Sulfate Fraction	1.09 (0.03)	89
60% Ammonium Sulfate Supernatant	0.19 (0.01)	15

[a] 1 unit is defined as the amount of enzyme which will generate 1 umole dopachrome/minute from a solution of 2.5 mM L-3,4-DOPA in 50 mM phosphate buffer, pH 6.8, containing 0.1% sodium cholate. Dopachrome was monitored spectroscopically at 475 nm.

[b] 6.7g of tumor tissue, which had been grown s.c. in C57BL/6J mice, was homogenized in 10 parts of 50 mM phosphate buffer, pH 6.8, containing 0.1% sodium cholate. The homogenate was then stirred for 30 minutes and sampled. Cellular debris was removed from the sample by microcentrifugation for 1 minute at 8,700 g before performing the dopachrome assay. The remainder of the homogenate was used without prior centrifugation for the ammonium sulfate step.

1978). Therefore, the B-16 model appears similar to human melanoma with regard to tyrosinase activity.

Characterization of Product

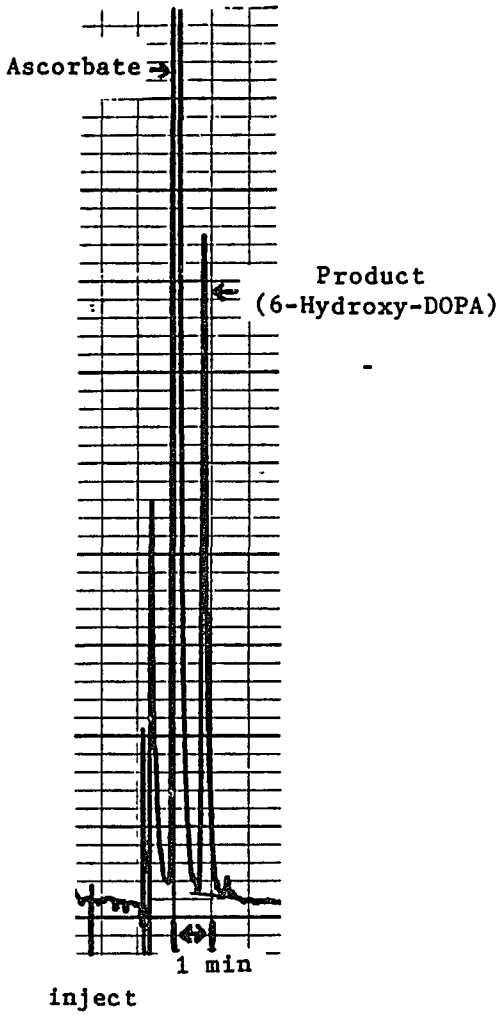
Experiments were performed to determine whether B-16 tyrosinase could hydroxylate 2,4-DOPA and generate 6-hydroxy-DOPA as product. When B-16 tyrosinase was added to a solution of 1 mM 2,4-DOPA in the presence of 50 uM L-3,4-DOPA as cosubstrate, a product was generated which co-chromatographed with a standard of 6-hydroxy-DOPA (Figure 3-11).

The product generated from 2,4-DOPA by B-16 tyrosinase was characterized further by determination of its electrochemical properties. Figure 3-12 shows the resultant plots of relative peak height versus electrode potential. The half-wave potential of enzyme-generated 6-hydroxy-DOPA was +0.30 volts versus a Ag/AgCl reference electrode, similar to that of a standard of 6-hydroxy-DOPA (+0.29 volts). For comparison, a standard of L-3,4-DOPA was included and had a half-wave potential of +0.59 volts. (Note: The values of half-wave potentials reported here for standards differ slightly from those in the previous experiment in section 3.1.1. Variations occur since the absolute half-wave potential depends upon the efficiency of the electrode, which in turn is dependent upon the final polishing of the glassy carbon electrode surface).

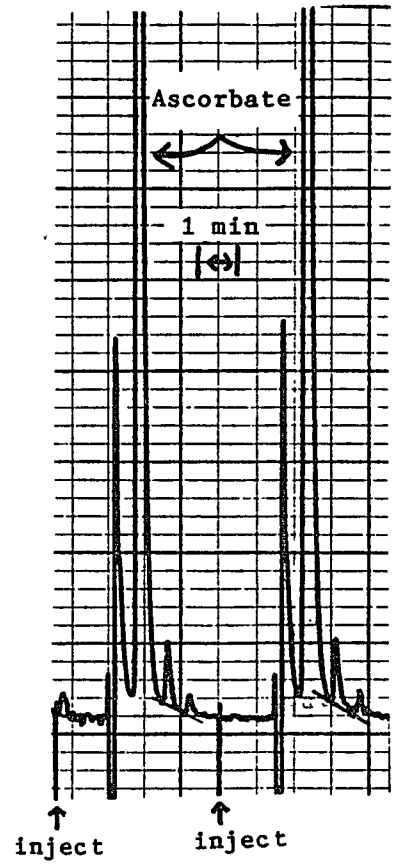
Figure 3-11. HPLC Chromatograms Demonstrating the Synthesis of 6-Hydroxy-DOPA by B-16 Tyrosinase

B-16 tyrosinase (6.721×10^{-4} units) was added to a final volume of 4.2 mLs of 50 mM phosphate buffer, pH 6.8, containing 1 mM 2,4-DOPA (substrate), 30 uM L-3,4-DOPA (cosubstrate), 0.1 mM ascorbate, and 100 uM DTPA. An aliquot was removed after 10 minutes, acidified, centrifuged, and analyzed by HPLC with electrochemical detection (electrode potential = +0.4 volts vs. a Ag/AgCl reference). 6-Hydroxy-DOPA (product) was separated from starting materials by a mobile phase of 50 mM phosphate buffer, pH 3.0, containing 1 mM EDTA, at a flow rate of 1.5 mLs/minute. Panel A demonstrates the enzymatic synthesis of 6-hydoxy-DOPA (1.35 uM in acidified sample). Ascorbate elutes in the void volume, before 6-hydroxy-DOPA, while L-3,4-DOPA and 2,4-DOPA elute after 6-hydroxy-DOPA. At the electrode potential used in this study, the L-3,4-DOPA and 2,4-DOPA do not give rise to significant peaks on the chromatogram, and they are not readily apparent. Panel B shows a chromatogram of control samples to which buffer was added in place of enzyme. A small, constant, peak can be seen immediately before the position at which 6-hydroxy-DOPA would elute. In both panels A and B, 20 uLs of sample were injected. Panel C demonstrates that the tyrosinase-generated product co-chromatographs with a standard of 6-hydroxy-DOPA. Tyrosinase-generated product (26.98 pmoles) and a standard of 6-hydroxy-DOPA (23.44 pmoles) were loaded into the same syringe and injected. The mixture gave rise to a single, smooth peak, thereby satisfying the criterion of co-chromatography.

Panel A

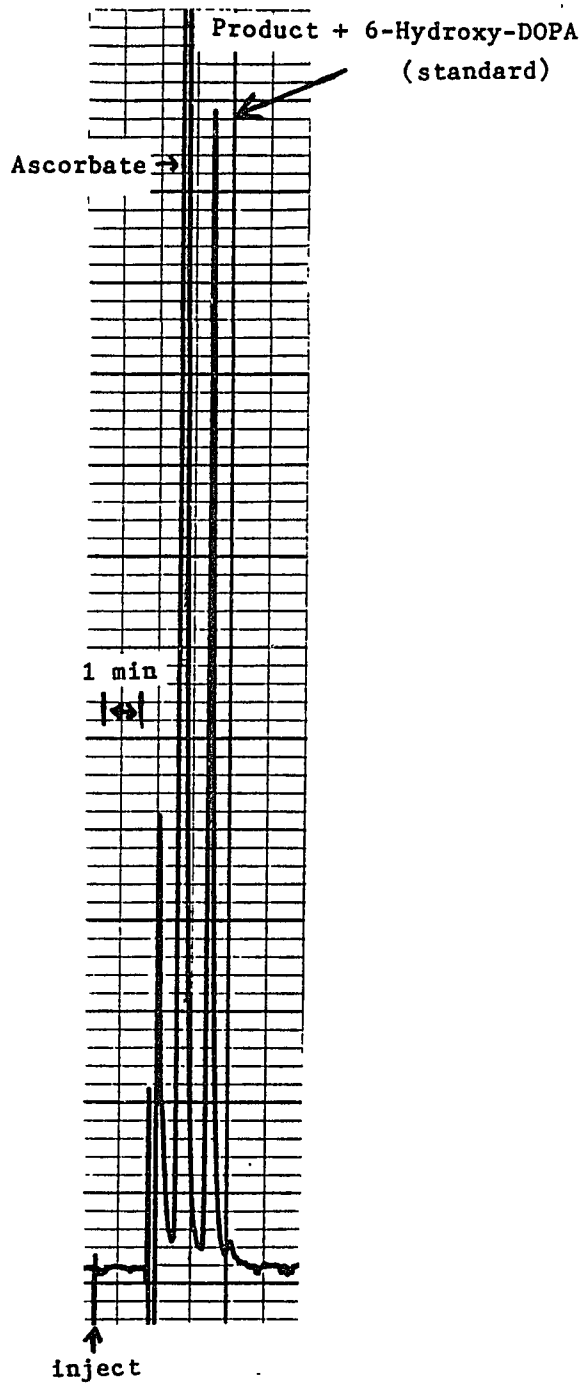


Panel B



Control (0 min) Control (12 min)

Panel C



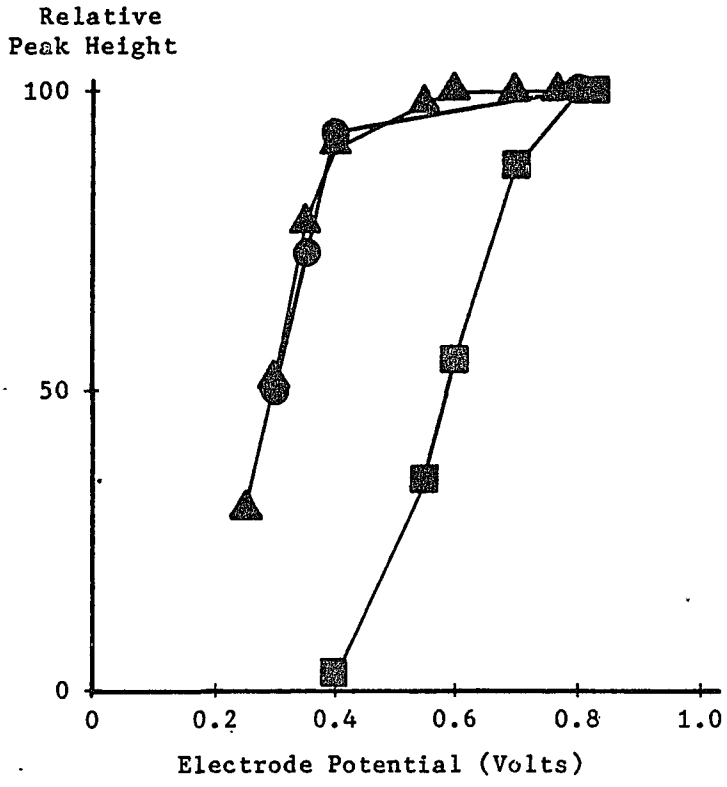


Figure 3-12. Electrochemical Oxidation Properties of Standards and B-16 Tyrosinase-Generated 6-Hydroxy-DOPA.

Product and standards were analyzed by HPLC with electrochemical detection. A mobile phase consisting of 50 mM phosphate buffer, pH 3.0, containing 1 mM EDTA was used at a flow rate of 1.0 mL/minute. Variation of the electrode potential between replicate injections allowed determination of the electrochemical oxidation properties of the product and standards at the pH of the mobile phase.

The relative peak heights are shown for tyrosinase-generated 6-hydroxy-DOPA (circles, 100 = 1.83 nAmps), authentic 6-hydroxy-DOPA (triangles, 100 = 2.67 nAmps), and L-3,4-DOPA (squares, 100 = 4.74 nAmps). The average standard deviation for duplicate determinations was 1.8 relative peak height units (range: 0.5 to 6.7).

Characteristics of the Reaction of 2,4-DOPA with B-16 Tyrosinase

Generation of 6-hydroxy-DOPA from 2,4-DOPA was blocked by addition of 100 uM PTU, an inhibitor of tyrosinase, thereby demonstrating the enzymatic nature of the reaction. When 2,4-DOPA was present at 5 mM with 50 uM L-3,4-DOPA as substrate, addition of 100 uM PTU inhibited the reaction by 93% (Table 3-1, experiment c).

The rate of accumulation of product was linear with time (Figure 3-13), and no consistent burst was apparent. Indeed, in some experiments, a definite lag (i.e., negative burst) was present (Table 3-5).

The metal-chelating agent, DTPA, was present in the reaction mixture to suppress metal-catalyzed autoxidation of product or ascorbate. To determine if DTPA had any effect upon tyrosinase activity, experiments were performed in the presence and absence of DTPA. The dopachrome assay was used to monitor activity. 0.1% sodium cholate was included in all buffers to keep the normally membrane-bound mammalian tyrosinase in solution. L-3,4-DOPA was present at 2.5 mM. The 40-60% ammonium sulfate fraction of B-16 tyrosinase was added to a final dilution of approximately 1%. In the absence of DTPA, the increase in absorbance at 475 nm was 0.016/min ($\pm$ 0.001 S.D., N = 3). When enzyme was preincubated for 10 minutes in buffer containing 100 uM DTPA and assayed in the presence of 100 uM DTPA, the increase in absorbance was 0.015/min ($\pm$ 0.002 S.D., N = 3). Therefore, it was concluded that DTPA did not affect the activity of B-16 tyrosinase during the time periods in which experiments were routinely run.

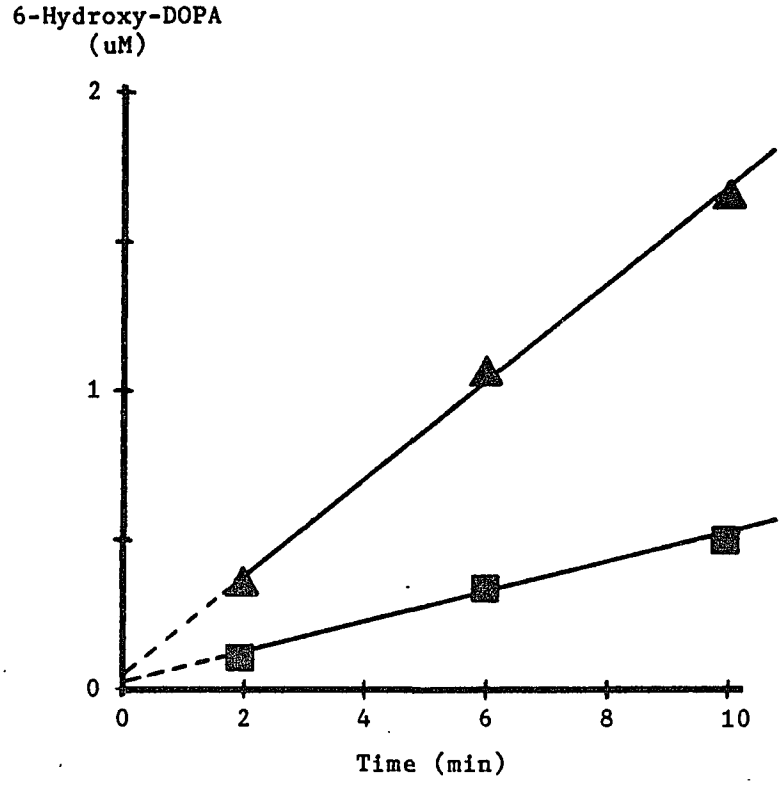


Figure 3-13. Generation of 6-Hydroxy-DOPA from 2,4-DOPA by B-16 Tyrosinase

2,4-DOPA was incubated with B-16 tyrosinase (7.812×10^{-4} units) in the presence of 0.1 mM ascorbate and either 1 uM (squares) or 10 uM (triangles) L-3,4-DOPA in 4.2 mLs of 50 mM phosphate buffer, pH 6.8, containing 100 uM DTPA. Timed aliquots were acidified, centrifuged, and analyzed for product by HPLC with electrochemical detection. The data were obtained from the experiments shown in Figure 3-15.

Table 3-5

Burst Effect when 2,4-DOPA Reacts with B-16 Tyrosinase

	2,4-DOPA (mM)	L-3,4-DOPA (uM)	Burst (S.D.) (umole/unit)
a	0.5	50	-0.56 (0.42)
	1.0	50	-0.84 (0.77)
	5.0	50	-3.52 (1.07)
b	1.0	1	0.44 (0.31)
	1.0	5	-0.06 (0.26)
	1.0	10	0.42 (0.35)
	1.0	50	0.00 (0.38)
	1.0	100	0.71 (0.11)

The formation of product was monitored by HPLC. The burst was determined by extrapolation of data points to zero time. A positive burst represents the amount of product rapidly formed before steady-state is acheived. A negative burst represents a "lag" before the reaction proceeds at the steady-state rate.

[a]The data are from the experiments shown in Figure 3-14.

[b]The data are from the experiments shown in Figure 3-15.

In some experiments, a crude homogenate of tyrosinase was used so that the actual dilution from the in vivo state would be known. At a final dilution of 200-fold, the rate of product accumulation was 34 nM/min (± 2 S.D., N = 4) in the presence of 1 mM 2,4-DOPA, 50 uM L-3,4-DOPA, 1 mM ascorbate, and 100 uM DTPA in 50 mM phosphate buffer, pH 6.8. This would imply that the rate of product formation in vivo would be in the range of uM/min (Morrison and Cohen 1983).

Kinetic Parameters and pH Dependence of the Reaction of 2,4-DOPA with B-16 Tyrosinase

Experiments were performed in which the concentration of either 2,4-DOPA or L-3,4-DOPA was varied in order to determine kinetic parameters. When substrate concentration was varied while holding L-3,4-DOPA concentration constant at 50 uM, the resultant double-reciprocal plot was linear (Figure 3-14) and yielded an apparent K_m of 3.71 mM and V_{max} of 4.43 umole/min/unit (Table 3-3).

When the concentration of L-3,4-DOPA was varied while holding the concentration of 2,4-DOPA constant at 1 mM, a stimulatory effect of the L-3,4-DOPA was evident (Figure 3-15). Double-reciprocal analysis yielded a $K_{activator}$ of 3.1 uM (Table 3-3). (Note: Since 100 uM L-3,4-DOPA produced approximately 28% inhibition compared to 50 uM L-3,4-DOPA, the 100 uM point was not used in the analysis).

The pH dependence of the reaction was determined using 1 mM 2,4-DOPA as substrate and 30 uM L-3,4-DOPA as cosubstrate. The pH maximum was found to be approximately 8 (Figure 3-16).

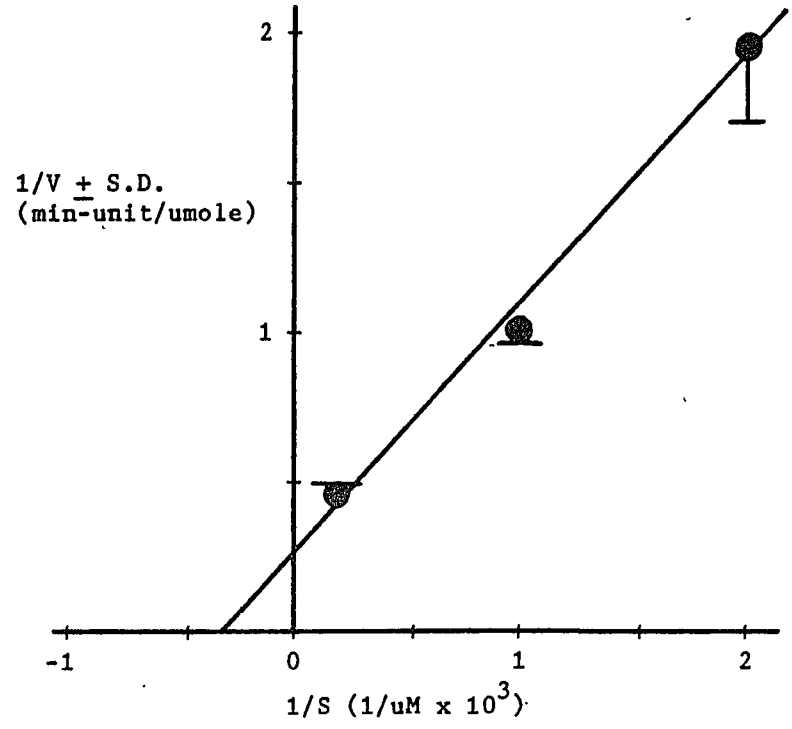


Figure 3-14. Conversion of 2,4-DOPA to 6-Hydroxy-DOPA by B-16 Tyrosinase; Double-Reciprocal Analysis

B-16 tyrosinase (1.428×10^{-4} units) was added to a final volume of 4.2 mLs of 50 mM phosphate buffer, pH 6.8, containing 50 uM L-3,4-DOPA (cosubstrate), 0.1 mM ascorbate, and 100 uM DTPA. Timed aliquots were acidified, centrifuged, and analyzed by HPLC with electrochemical detection. Each point represents the mean of 3 experiments.

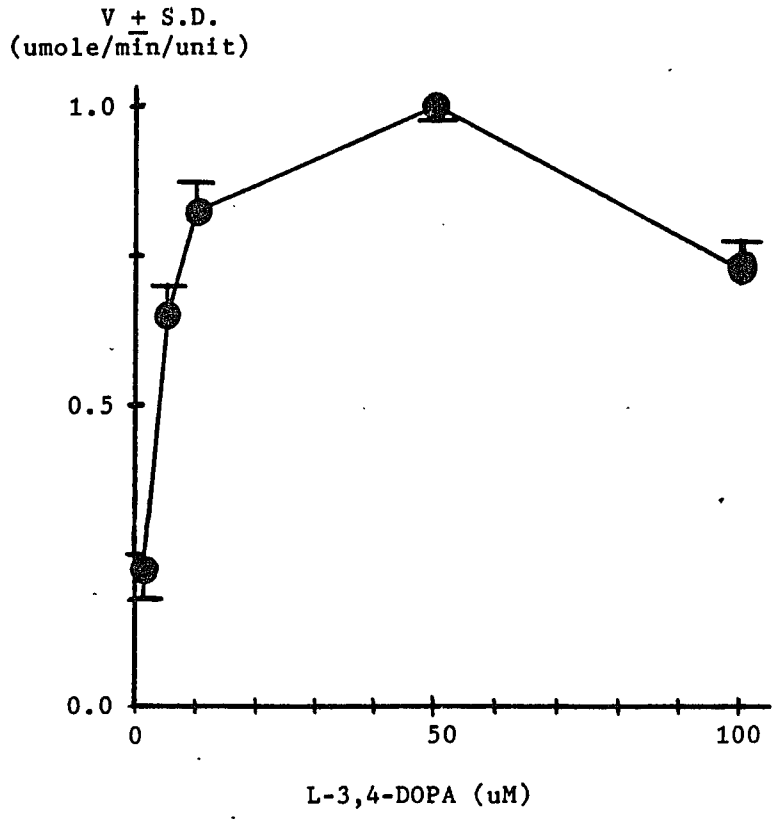


Figure 3-15. Characterization of L-3,4-DOPA as Cosubstrate for B-16 Tyrosinase-Catalyzed Conversion of 2,4-DOPA to 6-Hydroxy-DOPA

B-16 tyrosinase (7.812×10^{-4} units) was added to a final volume of 4.2 mLs of 50 mM phosphate buffer, pH 6.8, containing 0.1 mM ascorbate, 100 uM DTPA, and 1 mM 2,4-DOPA (N=3). Timed aliquots were acidified, centrifuged, and analyzed by HPLC with electrochemical detection.

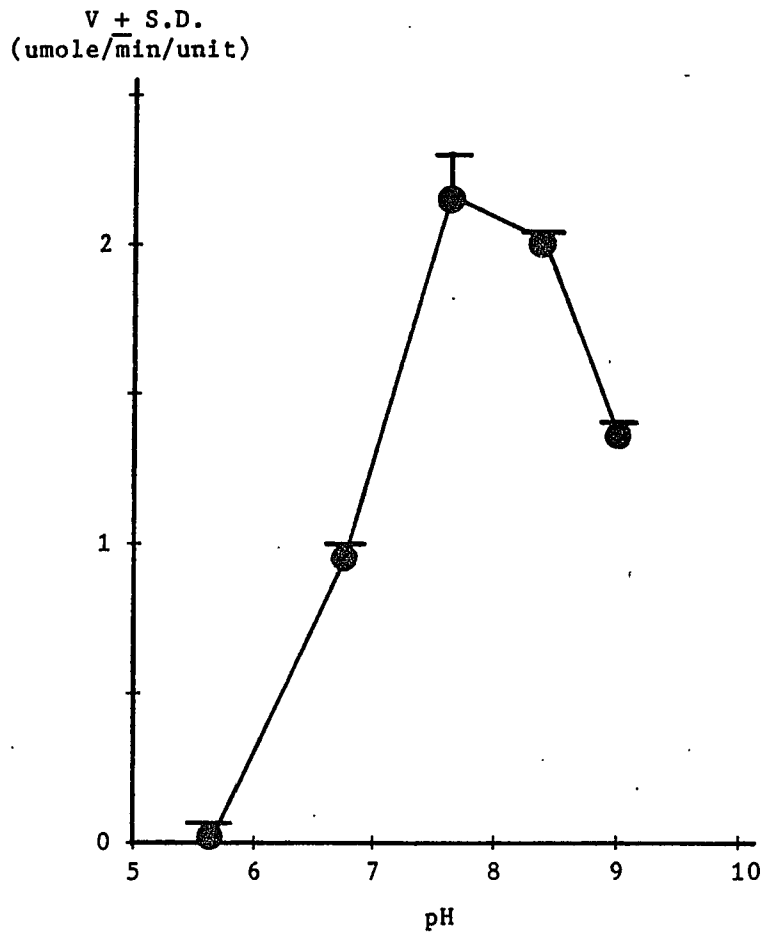


Figure 3-16. pH Dependence of the Conversion of 2,4-DOPA to 6-Hydroxy-DOPA by B-16 Tyrosinase

B-16 tyrosinase (6.721×10^{-4} units) was added to a final volume of 4.2 mLs of 50 mM buffer (see methods) containing 0.1 mM ascorbate, 100 uM DTPA, 30 uM L-3,4-DOPA (cosubstrate), and 1 mM 2,4-DOPA (N=2). Timed aliquots were acidified, centrifuged, and analyzed by HPLC with electrochemical detection.

3.2: DISCUSSION OF ENZYMATIC RESULTS

3.2.1: Characterization of Products

It has been reported by Hansson et al. (1980) that tyrosinase can hydroxylate L-3,4-DOPA in the 5-position to generate "5-hydroxy-DOPA;" no hydroxylation in the 6-position was seen. Consequently, it was predicted that the prodrugs, 2,4-DOPA and 2,4-dopamine, would also be hydroxylated in the 5-position (Figure 1-2) to generate 2,4,5-trihydroxyphenylalanine and 2,4,5-trihydroxyphenylethylamine, respectively (i.e., 6-hydroxy-DOPA and 6-hydroxydopamine, if one numbers the ring in the opposite direction). The hydroxylation of 2,4-DOPA was also confirmed for mammalian tyrosinase. The tyrosinase-generated products were shown to co-chromatograph with standards of 6-hydroxy-DOPA or 6-hydroxydopamine, respectively. Alternate products were the corresponding 2,3,4- and 2,4,6-trihydroxy compounds. Standards of 2,3,4- and 2,4,6-trihydroxyphenylethylamine were available and could be separated from tyrosinase-generated 6-hydroxydopamine. The alternate products were not observed in samples of 6-hydroxydopamine generated by mushroom tyrosinase.

The tyrosinase-generated products were also characterized electrochemically. The products were found to have half-wave potentials similar to the appropriate standards. Standards of the possible alternate 2,3,4- and 2,4,6-trihydroxy products were not available in the amino acid series. However, consideration of their expected electrochemical properties tends to rule out these alternate possibilities. 2,4,6-trihydroxyphenylalanine cannot form a quinone

upon oxidation and is, therefore, relatively stable. 2,3,4-Trihydroxyphenylalanine would be able to form a quinone upon oxidation, as would L-3,4-DOPA and 6-hydroxy-DOPA. The ability to form a quinone makes the oxidation of these compounds favorable. As a result, their half-wave potentials (qualitatively similar to the standard Nernst reduction potential) are easily determined by electrochemical methods. The standard Nernst reduction potentials of compounds similar to some of the theoretical products have been reported in the literature (Clark 1960): (a) 1,2,4-trihydroxybenzene, 0.599 volts (compound similar to 6-hydroxy-DOPA); (b) 1,2,3-trihydroxybenzene, 0.713 volts (compound similar to 2,3,4-trihydroxyphenylalanine); and (c) 1,2-dihydroxybenzene, 0.792 volts (compound similar to L-3,4-DOPA). On the basis of these values, 2,3,4-trihydroxyphenylalanine should exhibit a half-wave potential intermediate in value between that for 6-hydroxy-DOPA and L-3,4-DOPA. Since the half-wave potential of the product generated from 2,4-DOPA by either mushroom or mammalian tyrosinase was virtually identical to that of a standard of 6-hydroxy-DOPA, 2,3,4-trihydroxyphenylalanine can be ruled out as a major product of the reaction.

On the basis of co-chromatography with standards, similarity of electrochemical properties to those of standards, and separation from the theoretically alternate products in the amine series, the products generated by tyrosinase from 2,4-DOPA and 2,4-dopamine were concluded to be 6-hydroxy-DOPA and 6-hydroxydopamine, respectively.

3.2.2: Burst Effect with Mushroom Tyrosinase

In studies with mushroom tyrosinase, an initial burst of enzymatic activity was noted when either 2,4-DOPA or 2,4-dopamine were used as substrate. The burst varied directly with substrate and L-3,4-DOPA (cosubstrate) concentration. An apparent burst of activity might occur if the initially formed product were to very rapidly saturate an inhibitory allosteric site or modify the active site directly. In the latter case, the products, 6-hydroxy-DOPA or 6-hydroxydopamine, could theoretically generate oxy-radicals in the active site, thus causing damage which might modify binding of substrate and cosubstrate. Alternatively, the respective quinones might covalently add to residues present in or near the active site with a resultant decrease in substrate and cosubstrate binding. Although the burst effect is interesting on theoretical grounds, priority was given to determination of the steady-state kinetic parameters which are more relevant with regard to treatment of melanoma. However, it is hoped that future studies may elucidate the mechanism underlying the burst phenomenon.

3.2.3: Kinetic Considerations

Mushroom tyrosinase was initially used as a general model for tyrosinase. Mushroom tyrosinase is a soluble enzyme which exists in a polymeric state; a tetramer of 2 light chains and 2 heavy chains predominates. In contrast, mammalian tyrosinase exists predominantly as a membrane-bound monomer. Nonetheless, both mushroom tyrosinase and mammalian tyrosinase utilize the same reaction mechanism (Lerch

1981), and a comparison of the kinetic parameters of the reaction of tyrosinase with 2,4-DOPA demonstrated a remarkable similarity between the two enzymes (Table 3-3).

At pH 6.8, the K_m for 2,4-DOPA was 1.25 mM when mushroom tyrosinase was used and 3.71 mM when B-16 tyrosinase was used (L-3,4-DOPA was present at 50 uM in both studies). Although a K_m in the mM range is not ideal for in vivo chemotherapy, it is possible to obtain mM concentrations of 2,4-DOPA in vivo (see Appendix A and the discussion therein). In the present studies, D,L-2,4-DOPA was employed. In future studies, it may be reasonable to obtain L-2,4-DOPA for testing. It has been demonstrated (H. Rorsman, personal communication) that mammalian tyrosinase is not able to carry out the hydroxylation of D-p-tyrosine, but can only use the "L" enantiomer as substrate. It may, therefore, be the case that only L-2,4-DOPA will prove useful in chemotherapeutic trials. The "D" enantiomer might even be detrimental in that it might inhibit the hydroxylation of L-2,4-DOPA or even produce non-tyrosinase-mediated cytotoxicity as a side effect. These possibilities should be tested when pure L-2,4-DOPA and D-2,4-DOPA become available at a later date.

L-3,4-DOPA served as a cosubstrate for the reaction. At pH 6.8, in the presence of either 1 mM or 5 mM 2,4-DOPA, the $K_{activator}$ for L-3,4-DOPA as cosubstrate for mushroom tyrosinase was 2.5 uM and 1.3 uM, respectively. These results indicate that 2,4-DOPA did not compete with the cosubstrate function of L-3,4-DOPA over the concentration range examined. For B-16 tyrosinase at pH 6.8, the $K_{activator}$ for L-3,4-DOPA as cosubstrate was 3.1 uM in the presence of 1 mM 2,4-DOPA.

In experiments with hamster melanoma tissue, Pomerantz and Warner (1967) determined that the concentration of L-3,4-DOPA within the tumor ranged from 20-40 uM; therefore, physiological levels of L-3,4-DOPA are sufficient to activate the conversion of 2,4-DOPA to 6-hydroxy-DOPA.

The kinetic parameters of the conversion of 2,4-dopamine to 6-hydroxydopamine by mushroom tyrosinase were determined. The K_m for 2,4-dopamine was found to be 1.26 mM at pH 6.8 in the presence of 50 uM L-3,4-DOPA. When 2,4-dopamine was present at 1 mM, the $K_{activator}$ for L-3,4-DOPA was found to be 1.9 uM. Both the K_m and the $K_{activator}$ were, therefore, similar to the K_m and $K_{activator}$ determined when 2,4-DOPA was used as substrate (i.e., 1.25 mM and 2.5 uM, respectively). However, the V_{max} with 2,4-dopamine as substrate was 2.1-fold higher than when 2,4-DOPA was used as substrate.

Mushroom tyrosinase was used to further explore the kinetics of the reaction. With 2,4-DOPA as substrate, both the V_{max} and K_m increased when the concentration of L-3,4-DOPA was increased. An increase in K_m reflects poorer substrate binding which results in a decrease in rate. Since increasing the concentration of L-3,4-DOPA resulted in a faster rate of product accumulation, it was concluded that the increase in V_{max} was the dominant effect. Both L-3,4-DOPA and 6-hydroxy-DOPA (generated from 2,4-DOPA) are also substrates for tyrosinase and are oxidized to quinones (Graham and Jeffs 1977). Therefore, it is likely that the increase in K_m was due to competition with 2,4-DOPA at the active site by either L-3,4-DOPA and/or 6-hydroxy-DOPA.

3.3.4: Effect of pH

Examination of the pH dependence of the reaction of tyrosinase with 1 mM 2,4-DOPA in the presence of 30 uM L-3,4-DOPA as cosubstrate revealed that a pH maximum of 9 for mushroom tyrosinase, and a pH maximum of 8 for B-16 tyrosinase. Although the pH maxima were above physiological pH, the reactions still proceeded at reasonable rates at pH 7.3 (i.e., approximately 0.4- and 0.8-times maximal rate with mushroom and B-16 tyrosinase, respectively). It was important to assess the ability of tyrosinase to carry out the conversion of the prodrug at pH's below 7.3. In vivo, as solid tumors grow progressively larger, vessels become compressed and areas of central necrosis develop (Endrich et al. 1982). Due to hypoxia, viable cells adjacent to necrotic areas begin to produce excessive amounts of lactic acid and enter a state of metabolic acidosis; the lower pH limit reported for a state of metabolic acidosis is 6.8 (Altman and Dittmer 1964). The data indicate that both the mushroom enzyme and the B-16 enzyme will carry out the conversion of 2,4-DOPA to 6-hydroxy-DOPA at pH 6.8 and lower (Figures 3-11 and 3-17). At pH 6.8, the rates were approximately 0.3- and 0.4-times maximal for mushroom and B-16 tyrosinase, respectively.

When the pH dependence of the reaction of mushroom tyrosinase with 2,4-dopamine was examined, a broad maximum was found between pH 5.5 and 7.5 (cf. the sharp pH maximum of 9 for 2,4-DOPA as substrate with mushroom tyrosinase). Therefore, the greater V_{max} exhibited by 2,4-dopamine when compared to 2,4-DOPA at pH 6.8 may have been due to

the difference in pH maxima between the 2 substrates.

3.3.5: Conclusions

The data show that both mammalian and mushroom tyrosinase can carry out the synthesis of 6-hydroxy-DOPA from 2,4-DOPA. The synthesis of 6-hydroxydopamine from 2,4-dopamine was also demonstrated with mushroom tyrosinase. These observations set the stage for a chemotherapeutic trial with melanoma cells in culture.

By way of analogy to 6-hydroxy-DOPA, which crosses the blood-brain barrier, and 6-hydroxydopamine, which does not (Kostrzewa and Jacobowitz 1974), it is possible that the amino acid, 2,4-DOPA, might prove useful for the treatment of cerebral metastases of melanoma, in contrast to 2,4-dopamine which would not be expected to cross the blood-brain barrier. Therefore, 2,4-DOPA was chosen for trial in cell culture.

Chapter 4: CELL CULTURE STUDIES: RESULTS AND DISCUSSION

4.1: RESULTS

4.1.1: Treatment with 2,4-DOPA: Effect upon Cell Survival

B-16 Melanoma Cells

A preliminary trial (Table 4-1) demonstrated that continuous treatment of stationary $B\text{-}16_1$ cultures with 2,4-DOPA produced a cytotoxic effect. Cultures were treated with either 1, 3, or 5 mM 2,4-DOPA over a 3-day period. On days 2 and 3, the number of viable cells per culture was determined in duplicate. The data of Table 4-1 show that the cytotoxic effect was dose dependent. By day 2, cell number decreased to 67 % of control in cultures treated with 5 mM 2,4-DOPA, compared to 95 % of control in cultures treated with 1 mM 2,4-DOPA. However, the effect seemed to plateau and was similar after 2 or 3 days of treatment.

Although heavily melanized in vivo, subpopulations of melanoma cells lose the capacity to synthesize melanin in vitro. As a result, the melanoma cultures used in all studies reported here were a mixture of melanized and non-melanized cells. However, it is the melanized cells which provide the appropriate model for the heavily melanized state seen in vivo (Figure 4-1, panel A), and these cells were monitored in subsequent experiments. Since the melanized cells contain active tyrosinase, it was predicted that they might be preferentially affected by 2,4-DOPA treatment compared to the non-

TABLE 4-1

Cytotoxicty of 2,4-DOPA Against B-16_1 Melanoma Cells

Days of Treatment	2,4-DOPA (mM)	10^5 Cells (S.D.)	% of Control
2	0	7.85 (0.07)	100.0
	1	7.45 (0.07)	94.9
	3	5.48 (0.32)	69.8
	5	5.25 (0.28)	66.9
3	0	6.30 (0.71)	100.0
	1	6.03 (1.52)	95.7
	3	5.15 (0.50)	81.7
	5	4.38 (0.04)	69.5

Cultures were continuously treated with 1-5 mM 2,4-DOPA for either 2 or 3 days. Viability was assessed by exclusion of trypan blue. Cell counts were performed in duplicate and represent total cells (i.e., both melanized and non-melanized).

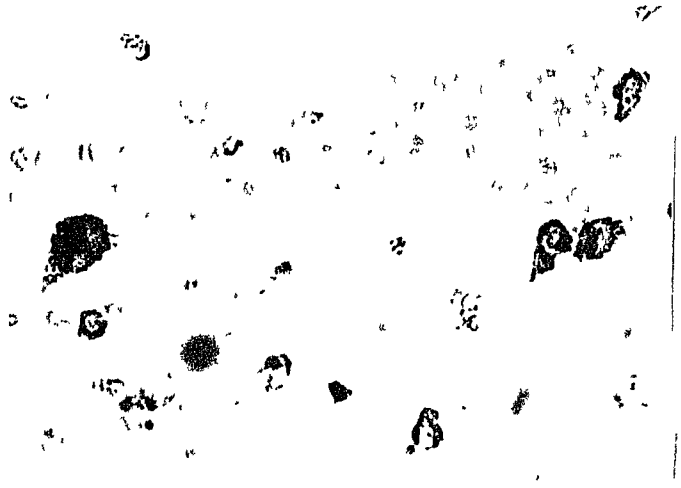
Figure 4-1. Photomicrographs of B-16 Melanoma in Vivo, Control $B\text{-}16_2$ Cultures, and 2,4-DOPA-Treated Cultures

Panel A shows a photomicrograph (200 x) of B-16 melanoma cells which had been grown as a subcutaneous tumor in C57BL/6J mice. The tumor tissue was excised and treated with trypsin to obtain a cell suspension. The cells were not stained. The melanoma cells can be seen to contain dark granules (melanosomes) laden with melanin pigment. Red blood cells (approximately 1/10 the size of the melanoma cells) and much cell debris (from necrotic areas within the tumor) are evident.

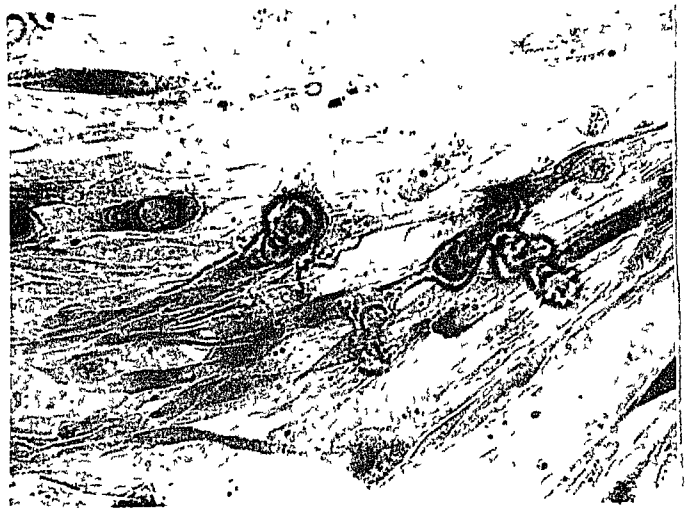
Panel B shows a photomicrograph (400 x) of $B\text{-}16_2$ melanoma cultures (control for day 7 of experiment). Cells were stained with L-3,4-DOPA and counterstained with Nile Blue (to accentuate melanin and lightly stain the remainder of the cell for visualization). The culture contains both melanized and non-melanized cells.

Panel C shows a photomicrograph (400 x) of parallel $B\text{-}16_2$ melanoma cultures after 7 days of treatment with 5 mM 2,4-DOPA. Cells were stained with L-3,4-DOPA and Nile Blue. Most of the melanized cells have disappeared from the culture.

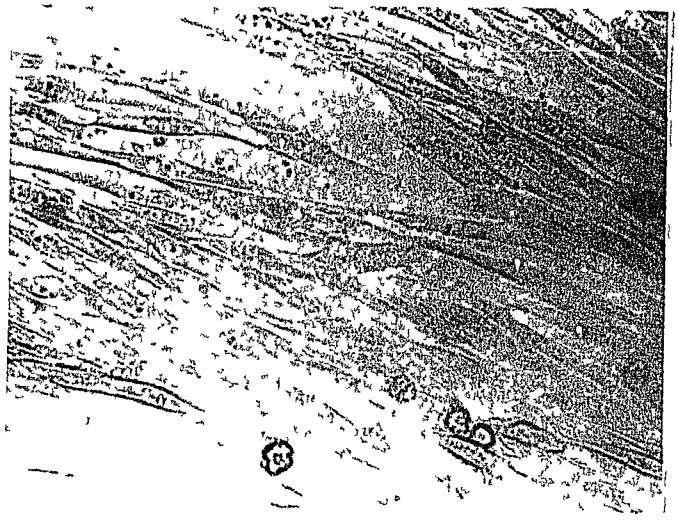
Panel A



Panel B



Panel C



melanized cells in the cultures. $B\text{-}16_2$ cultures, derived from the tumor tissue used in panel A above, were treated for 1 week with 5 mM 2,4-DOPA. The photomicrographs in Figure 4-1, panels B and C, demonstrate that treatment with 2,4-DOPA results in a marked reduction in the number of melanized cells.

In the experiment shown in Table 4-2, treatment of $B\text{-}16_1$ cells was initiated while the cultures were in the growth phase. During the growth phase, the number of cells increases with time. Cultures were continuously treated with 2.5 or 5 mM 2,4-DOPA during the 3-day trial period. On day 1, a significant cytotoxic effect was observed against the total population of cells in cultures treated with either dose of 2,4-DOPA. 2,4-DOPA at 5 mM reduced the number of viable cells to 68 % of control, and a dose of 2.5 mM decreased the population to 78 % of control.

The cytotoxic effect of 2,4-DOPA diminished as the cultures approached and reached saturation density (i.e., cell number reached a plateau) on days 2 and 3. However, examination of the melanized subpopulation revealed that the cytotoxicity of 2,4-DOPA was stronger against the melanized cells. Furthermore, the melanized cell number remained suppressed over the 3-day trial period. A dose of 5 mM 2,4-DOPA decreased the number of melanized cells to 25-37% of control over the 3-day period, and a dose of 2.5 mM 2,4-DOPA reduced the number of melanized cells to 46-57% of control. Since the melanized subpopulation remained suppressed throughout the treatment period, it was apparent that the loss of effect against the total population was due to the continued growth of non-melanized cells in treated cultures

Table 4-2

Cytotoxicty of 2,4-DOPA Against $B\text{-}16_1$ Melanoma Cells

Days of Treatment	2,4-DOPA (mM)	10^5 Total Cells (S.D.)	% of Control	Melanized[a] Cells (S.D.)	% of Control
0	0.0	3.62 (0.25)	100	80 (4)	100
1	0.0	7.67 (0.43)	100	117 (7)	100
	2.5	5.98 (0.39) b	78	60 (7) b	51
	5.0	5.22 (0.21) b	68	29 (2) b	25
2	0.0	9.10 (2.36)	100	119 (7)	100
	2.5	8.22 (0.99)	90	55 (8) b	46
	5.0	7.23 (0.24)	79	38 (1) b	32
3	0.0	8.83 (0.58)	100	93 (5)	100
	2.5	9.47 (0.83)	107	53 (6) b	57
	5.0	9.60 (1.33)	109	34 (5) b	37

Triplicate cultures were continuously treated with either 2.5 mM or 5 mM 2,4-DOPA for 1 - 3 days. Total viable cells (both melanized and non-melanized) were counted. Exclusion of trypan blue was used to assess viability. Parallel cultures were grown on coverslips. These cultures were stained for tyrosinase activity by incubation of fixed cells with L-3,4-DOPA, then counterstained with Nile blue.

[a]Melanized cells (stained for tyrosinase) per microscopic field (200x), N=3.

[b]$P < 0.01$, 1-tailed Dunnett's test. For all other points, $P > 0.05$.

until saturation density was reached. However, this occurred later in treated cultures than in control cultures. These results indicate that 2,4-DOPA is targeted primarily to the melanized subpopulation of cells.

Figure 4-2 shows the results of an experiment in which $B\text{-}16_1$ cells were continuously treated with 1, 2.5, or 5 mM 2,4-DOPA over a 7-day period. Treatment was begun soon after the cultures had reached saturation density. A strong cytotoxic effect against the whole culture (Figure 4-2, panel A) was evident as early as 1 day after initiation of treatment with either 2.5 or 5 mM 2,4-DOPA. When cultures were treated with 5 mM 2,4-DOPA, the cell number dropped to 51% of control on day 1 and then remained at approximately 40% for the following 6 days. Treatment with 2.5 mM 2,4-DOPA caused the cell number to drop to 77% of control on day 1 and slowly reach a plateau at approximately 55% of control. At 1 mM, 2,4-DOPA had no apparent effect against the whole culture on day 1; but by day 4, the cell number had dropped to 70% of control.

The effect of 2,4-DOPA upon the melanized and non-melanized subpopulations is shown in Figure 4-2, panels B and C, respectively. The data demonstrate that the melanized subpopulation was significantly more susceptible to the cytotoxic action of 2,4-DOPA than was the non-melanized subpopulation ($P < 0.0005$, 1-tailed t-test of % of control, paired by day of treatment and dose of 2,4-DOPA). For example, at a dose of 5 mM, the melanized cells decreased to an average of 7% of control over the 7-day trial period, while the non-melanized cells decreased to approximately 53% of control.

Figure 4-2. Cytotoxicity of 2,4-DOPA Against B-16_1 Melanoma Cells

Cultures were continuously treated with either 1, 2.5, or 5 mM 2,4-DOPA for 1 to 7 days. Controls received medium without prodrug. Cells were released from duplicate monolayer cultures by incubation with trypsin/EDTA. Aliquots of the resultant cell suspensions were added to trypan blue, and the number of viable cells/culture was determined. Panel A shows the effect of 2,4-DOPA treatment upon the total population of cells. The average standard deviation for duplicate cell counts was 0.11×10^5 cells (range: 0.01 to 0.49).

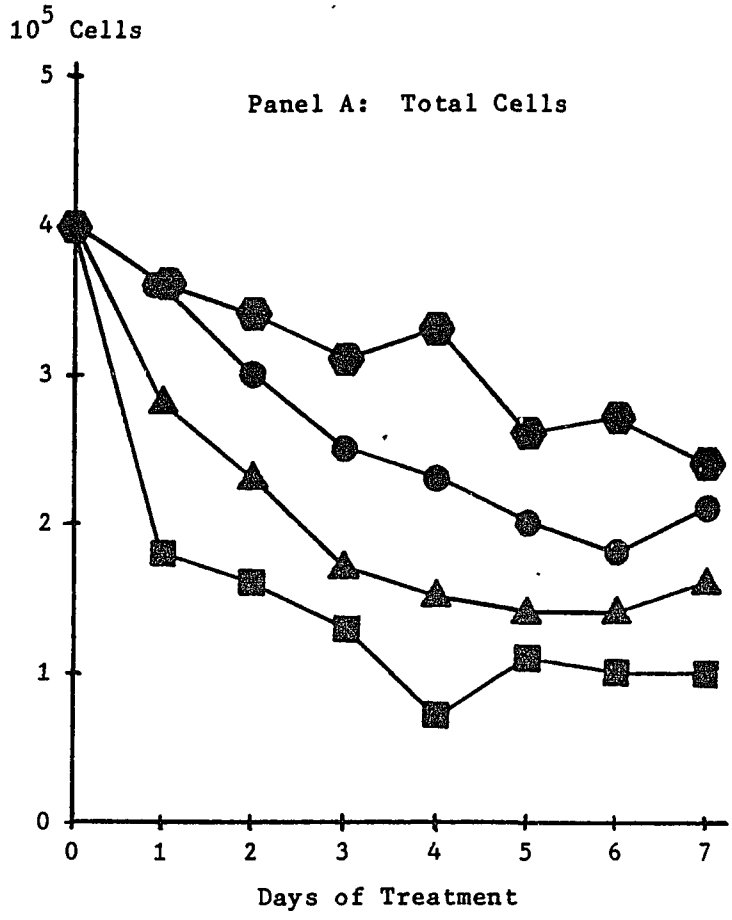
Aliquots of cells were also fixed in suspension and stained by incubation with L-3,4-DOPA. The stained cells were then scored in singlicate samples as melanized or non-melanized (see Methods). Panels B and C show the effect of 2,4-DOPA upon the melanized and non-melanized subpopulations of cells, respectively.

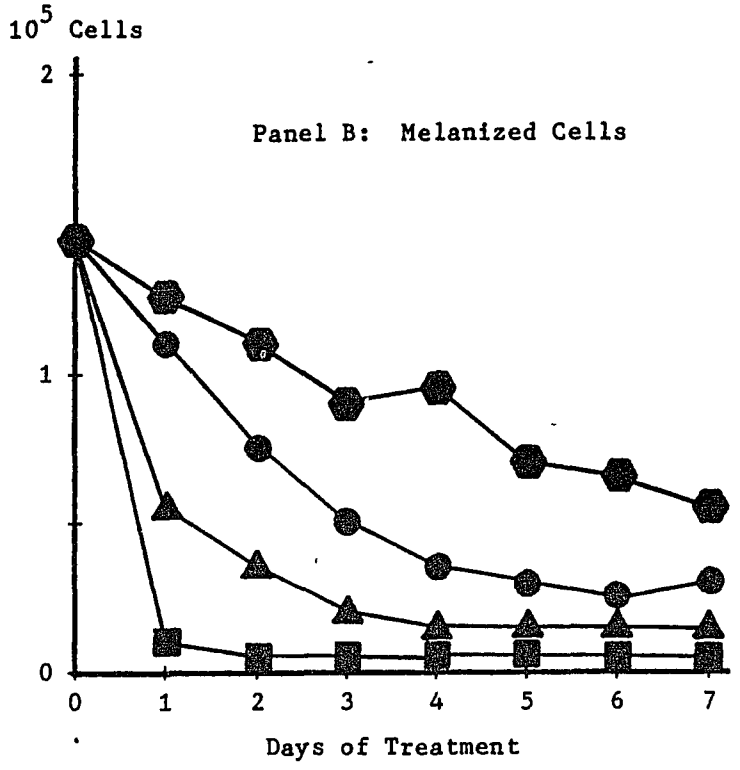
Hexagon = control

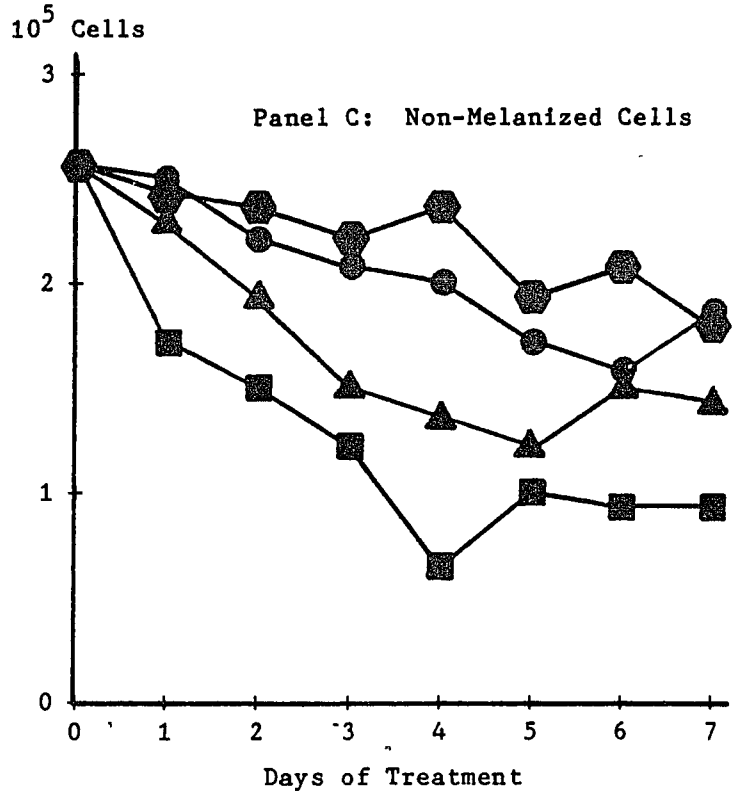
Circle = 1 mM 2,4-DOPA

Triangle = 2.5 mM 2,4-DOPA

Square = 5 mM 2,4-DOPA


10^5 Cells
Panel A: Total Cells
5
4
3
2
1
0
0
1
2
3
4
5
6
7
Days of Treatment


10^5 Cells
2
1
0
Panel B: Melanized Cells
0
1
2
3
4
5
6
7
Days of Treatment


10^5 Cells
3
2
1
0
Panel C: Non-Melanized Cells
0
1
2
3
4
5
6
7
Days of Treatment

$B\text{-}16_2$ cultures were used in the following studies. Studies with the $B\text{-}16_2$ cultures differed qualitatively from studies with $B\text{-}16_1$ cultures. The $B\text{-}16_2$ cultures continued to replicate during experimental trials, whereas the $B\text{-}16_1$ cultures either reached saturation density during the experiment or were used after entering stationary phase (i.e., no further replication).

Treatment of $B\text{-}16_2$ cultures with 2,4-DOPA resulted in dose-dependent cytotoxicity directed against the culture as a whole over the 7-day trial period (Figure 4-3, panel A). A significant cytotoxic effect was seen at all doses of 2,4-DOPA as early as one day after exposure to 2,4-DOPA ($P < 0.01$, 1-tailed Dunnett's test). The number of cells in control cultures increased gradually and was 2.9-times the initial cell number by day 7. Cultures treated with 1 mM 2,4-DOPA grew more slowly, and by day 7, the cell number increased 2.4-times. Cell growth was almost completely inhibited when cultures were treated with 2.5 mM 2,4-DOPA. Treatment with 5 mM 2,4-DOPA resulted in a decrease in total cell number to 65% of the initial day 0 value.

The number of melanized cells in control cultures increased over the 7-day period to approximately 2.9-times the initial day 0 value (Figure 4-3, panel B). Although the number of melanized cells initially decreased in cultures treated with 1 mM 2,4-DOPA, they subsequently continued to proliferate and by day 7 had increased to 1.8-times the initial number seen on day 0. The number of melanized cells in cultures treated with 2.5 mM 2,4-DOPA decreased within 24 hours of treatment and showed no signs of proliferation during the trial period. 5 mM 2,4-DOPA produced a continuous decrease in the

Figure 4-3. Cytotoxicity of 2,4-DOPA Against B-16_2 Melanoma Cells

Cultures were continuously treated with either 1, 2.5, or 5 mM 2,4-DOPA for 1 to 7 days. Controls received medium without prodrug. Cells were released from triplicate monolayer cultures by incubation with trypsin/EDTA. Aliquots of the resultant cell suspensions were added to trypan blue, and the number of viable cells/culture was determined. Panel A shows the effect of 2,4-DOPA upon the total cell population.

Aliquots of cells were also fixed in suspension and stained with L-3,4-DOPA. The stained cells were then scored in triplicate samples as melanized or non-melanized (see Methods). Panels B and C show the effect of 2,4-DOPA treatment upon the melanized and non-melanized subpopulations of cells, respectively.

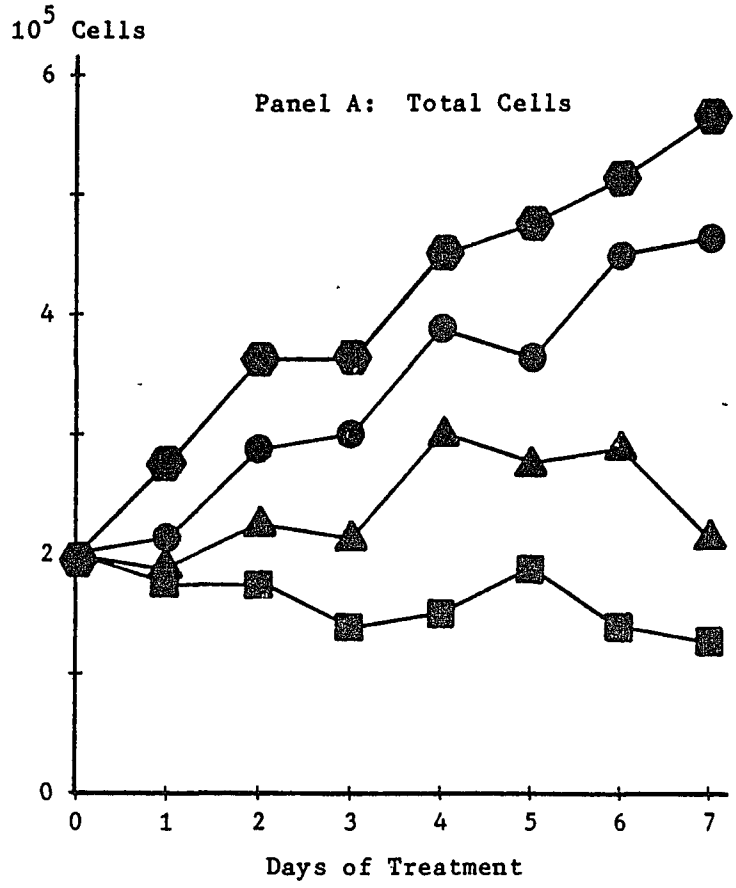
$P < 0.01$ (1-tailed Dunnett's test) for all points except: panel A, 1 mM dose on days 4 and 6 ($P < 0.05$); panel B, 1 mM dose on day 5 ($P < 0.05$); panel C, 1 mM dose on days 2 and 7 ($P < 0.05$) and on days 3, 4, and 6 ($P > 0.05$), 2.5 mM dose on day 4 ($P < 0.05$).

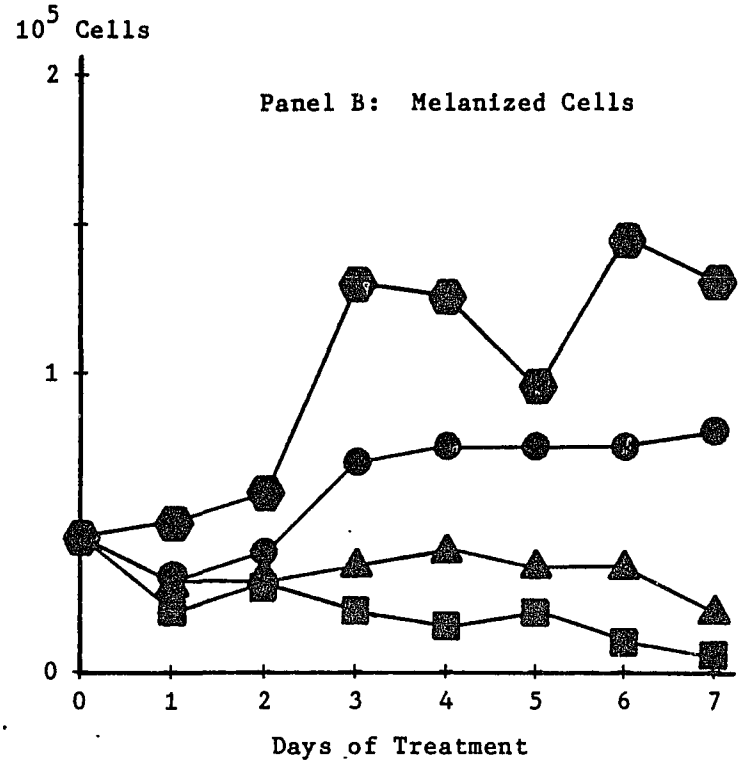
Hexagon = control

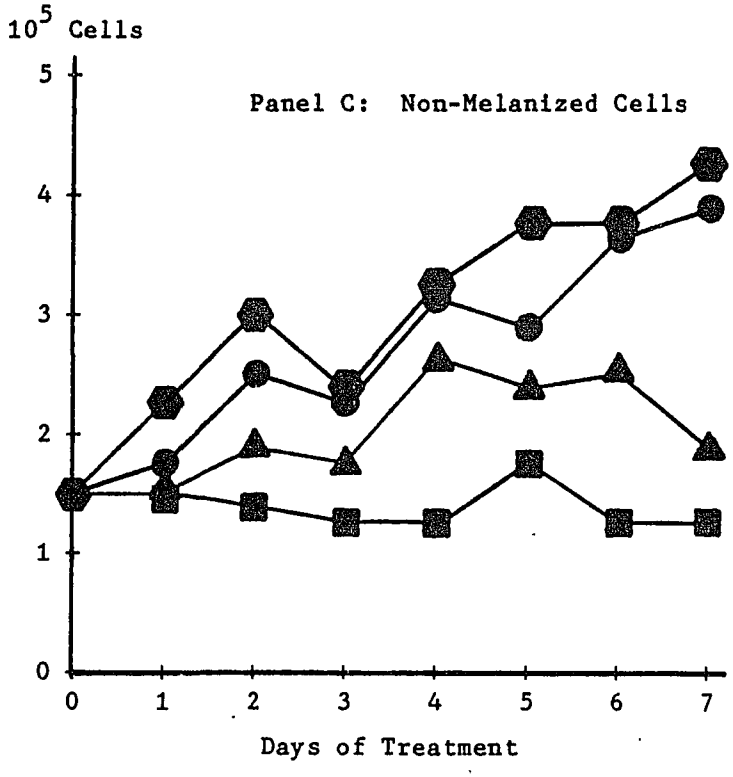
Circle = 1 mM 2,4-DOPA

Triangle = 2.5 mM 2,4-DOPA

Square = 5 mM 2,4-DOPA


10^5 Cells
6
4
2
0
Panel A: Total Cells
0
1
2
3
4
5
6
7
Days of Treatment


10^5 Cells
2
1
0
Panel B: Melanized Cells
0
1
2
3
4
5
6
7
Days of Treatment


10^5 Cells
5
4
3
2
1
0
Panel C: Non-Melanized Cells
0
1
2
3
4
5
6
7
Days of Treatment

number of melanized cells over the entire trial period so that, by day 7, treated cultures contained only 13% of the number of cells initially present on day 0.

The cytotoxicity directed against the non-melanized cells (Figure 4-3, panel C) was less than that against the melanized cells (panel B). The number of non-melanized cells in control cultures increased over the 7-day period to approximately 2.9-times the initial number seen on day 0. 1 mM 2,4-DOPA had little effect, and the non-melanized cells continued to grow over the 7-day period to reach 2.6-times the initial value. Cultures treated with 2.5 mM 2,4-DOPA grew slowly and reached a plateau at approximately 1.7-times the number initially present. Treatment with 5 mM 2,4-DOPA prevented growth of non-melanized cells, and by day 7, resulted in a decrease to 81% of the initial cell number.

Comparison of panels B and C in Figure 4-3, reveals that 2,4-DOPA treatment resulted in greater cytotoxicity against melanized cells than against non-melanized cells. The difference in the effect was significant ($P < 0.0005$, 1-tailed paired t-test comparing the percentage of control for melanized and non-melanized cells on each day for each dose).

Repetition of the above experiment (Table 4-3) again demonstrated that the melanized cells were more susceptible to 2,4-DOPA-mediated cytotoxicity than non-melanized cells. The cytotoxic effect against the melanized subpopulation was significantly greater than against the non-melanized population ($0.0005 < P < 0.005$, 1-tailed paired t-test comparing the percentage of control for melanized and non-melanized

Table 4-3

Cytotoxicity of 2,4-DOPA Against B-16$_2$ Melanoma Cells

Days of Treatment	2,4-DOPA (mM)	10^5 Total Cells (S.D.)	% of Control	10^5 Melanized Cells (S.D.)	% of Control	10^5 Non-Melanized Cells (S.D.)	% of Control
0	0.0	4.09 (0.27)	100.0	0.51 (0.06)	100.0	3.58 (0.24)	100.0
1	0.0	3.44 (0.42)	100.0	0.25 (0.03)	100.0	3.19 (0.39)	100.0
	1.0	3.00 (0.05)	87.2	0.19 (0.01) b	75.3	2.81 (0.05)	88.2
	a 2.5	2.10 (0.04) b	61.0	0.16 (0.02) b	65.2	1.94 (0.04) b	60.7
	5.0	1.83 (0.48) b	53.2	0.13 (0.02) b	50.3	1.70 (0.45) b	53.4
4	0.0	6.08 (0.62)	100.0	1.87 (0.20)	100.0	4.21 (0.43)	100.0
	1.0	4.60 (0.50) b	75.7	0.71 (0.15) b	38.2	3.89 (0.44)	92.3
	a 2.5	2.93 (0.42) b	48.2	0.29 (0.07) b	15.7	2.64 (0.38) b	62.6
	5.0	2.42 (0.08) b	39.8	0.14 (0.02) b	7.3	2.28 (0.08) b	54.2
7	a 0.0	7.29 (0.34)	100.0	1.59 (0.27)	100.0	5.70 (0.37)	100.0
	1.0	4.08 (0.37) b	56.0	0.31 (0.03) b	19.3	3.77 (0.34) b	66.2
	a 2.5	4.36 (0.08) b	59.8	0.23 (0.00) b	14.5	4.13 (0.08) b	72.4
	5.0	3.53 (0.29) b	48.4	0.10 (0.03) b	6.2	3.43 (0.28) b	60.2

Cultures were continuously treated with 2,4-DOPA. Cells were released from monolayers for determination of total cell count. Viability was assessed by exclusion of trypan blue. Cells were also stained in suspension with L-3,4-DOPA for scoring as melanized or non-melanized.

[a] N=2; N=3 for all other points.

[b] $P < 0.01$, 1-tailed Dunnett's test. For all other points, $P > 0.05$.

cells on each day for each dose). For example, by day 7, the melanized cells in cultures treated with 5 mM 2,4-DOPA had decreased to 20% of the initial number seen on day 0, while the non-melanized cells had only decreased to 96% of the initial value.

Cloudman Melanoma Cells

The cytotoxicity of 2,4-DOPA was also assessed against Cloudman melanoma cultures. The growth characteristics of the Cloudman cultures were similar to the $B\text{-}16_1$ melanoma cultures used in initial experiments. The Cloudman cultures reached saturation density rapidly, allowing the more slowly growing treated cultures to reach control cell number at later time points in the studies (see below).

Photomicrographs were prepared of control cultures and parallel cultures which had been treated for 4 days with 5 mM 2,4-DOPA (Figure 4-4). As was the case when B-16 cultures were treated with 2,4-DOPA, the treated Cloudman cultures contained far fewer melanized cells than control cultures.

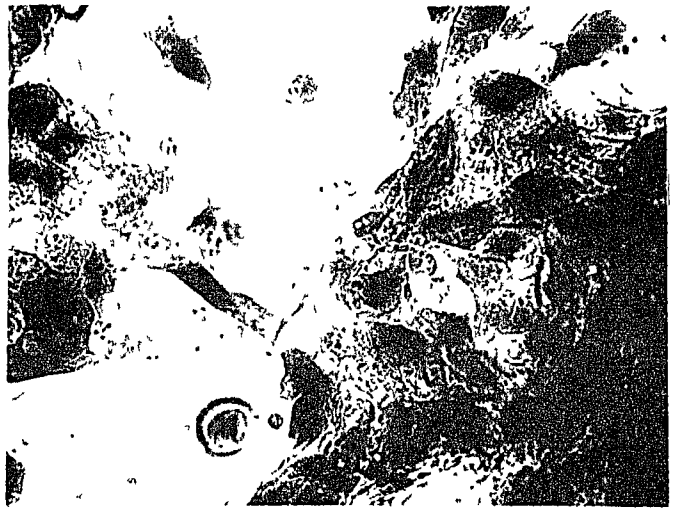
Continuous treatment of Cloudman melanoma cultures with 2,4-DOPA produced a strong cytotoxic effect against the whole culture (Figure 4-5 panel A). Treatment with 5 mM 2,4-DOPA effectively prevented the growth of the cultures throughout the 7-day trial period. Dose-dependent suppression of growth was initially observed during days 1 and 2 of treatment in cultures exposed to either 1 or 2.5 mM 2,4-DOPA. However, the difference between these two doses was not observed after day 3, and further treatment with 1 or 2.5 mM 2,4-DOPA had no apparent effect. As discussed below, however, cytotoxicity was evident against

Figure 4-4. Photomicrographs of Control and 2,4-DOPA-Treated Cloudman Melanoma

Panel A shows a photomicrograph (400 x) of untreated Cloudman melanoma cultures (4 days). Cells were stained with L-3,4-DOPA and counterstained with Nile Blue (to accentuate melanin and lightly stain the remainder of the cell for visualization). The culture contains predominantly melanized cells plus a few non-melanized cells.

Panel B shows a photomicrograph (400 x) of parallel Cloudman melanoma cultures after 4 days of treatment with 5 mM 2,4-DOPA. Cells were stained with L-3,4-DOPA and Nile Blue. Most of the heavily melanized cells have disappeared from the culture leaving predominantly non-melanized and lightly melanized cells.

Panel A



Panel B

Figure 4-5. Cytotoxicity of 2,4-DOPA Against Cloudman Melanoma Cells

Cultures were continuously treated with either 1, 2.5, or 5 mM 2,4-DOPA for 1 to 7 days. Controls received medium without prodrug. Cells were released from triplicate monolayer cultures by incubation with trypsin/EDTA. Aliquots of the resultant cell suspensions were added to trypan blue, and the number of viable cells/culture was determined. Panel A shows the effect of 2,4-DOPA upon the total cell population.

Aliquots of cells were also fixed in suspension and stained with L-3,4-DOPA. The stained cells were then scored in triplicate samples as melanized or non-melanized (see Methods). Panels B and C show the effect of 2,4-DOPA treatment upon the melanized and non-melanized subpopulations of cells, respectively.

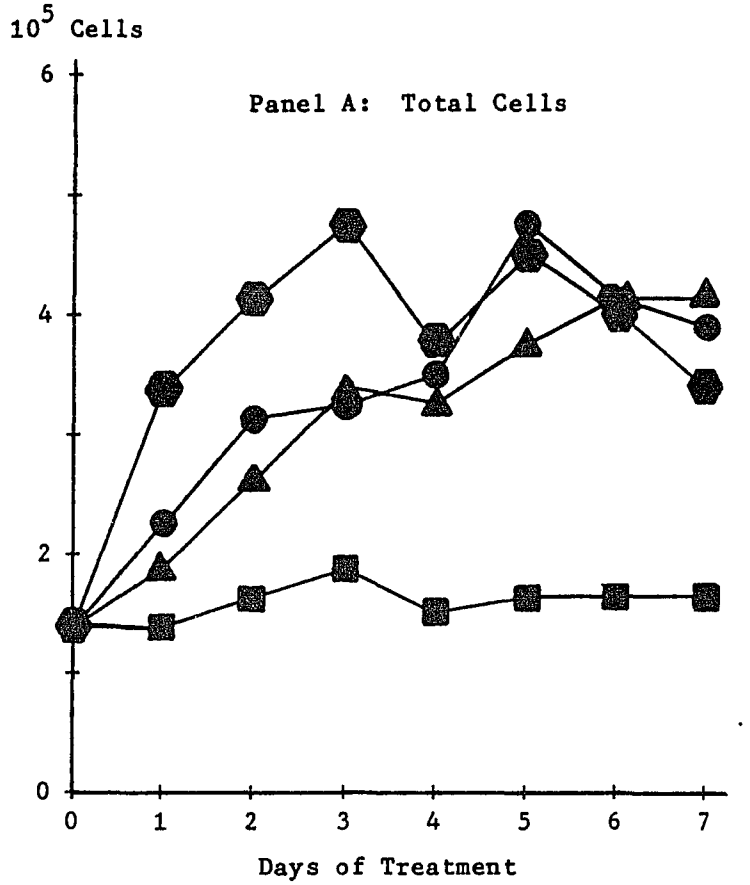
$P < 0.01$ (1-tailed Dunnett's test in panels A and B, 2-tailed Dunnett's test in panel C for all points except: panel A, 1 and 2.5 mM doses on days 4-7 ($P > 0.05$); panel B, 1 mM dose on days 2 and 6 ($P < 0.05$), 2.5 mM dose on day 2 ($P < 0.05$); panel C, 1 mM dose on days 3 and 4 ($P < 0.05$) and on day 6 ($P > 0.05$), 2.5 mM dose on days 3 and 6 ($P < 0.05$) and on days 4 and 5 ($P > 0.05$).

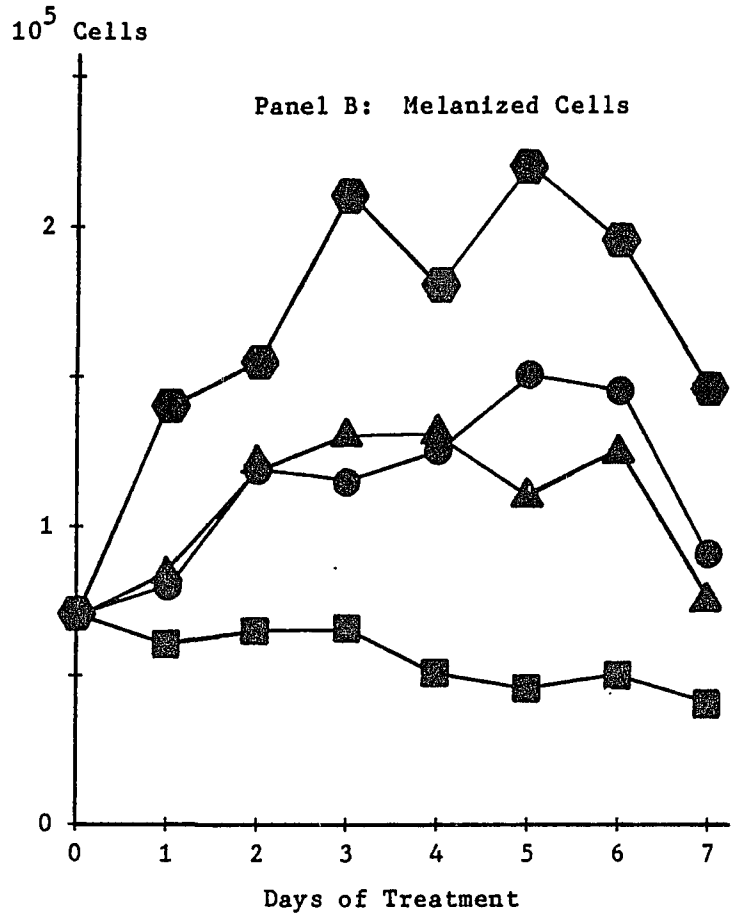
Hexagon = control

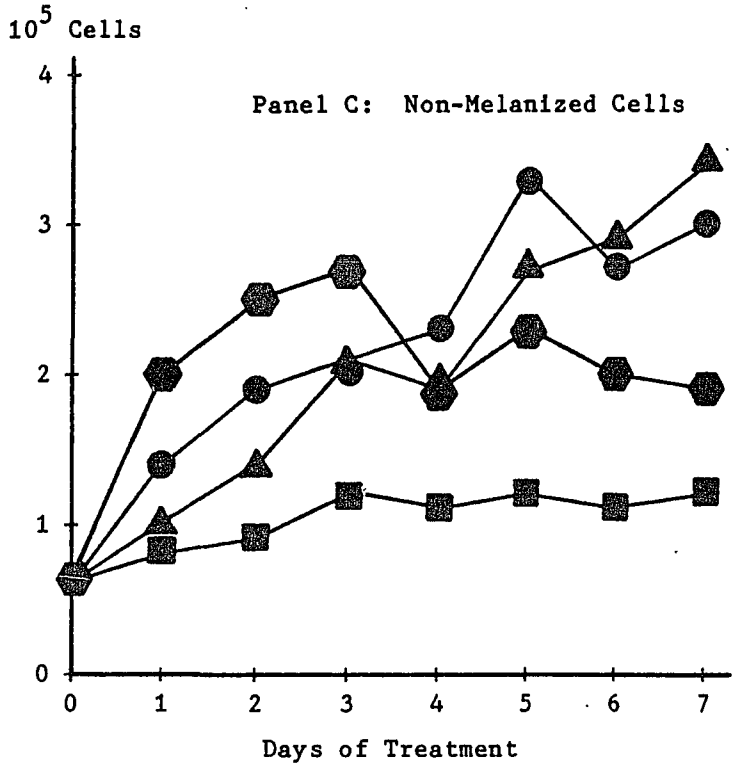
Circle = 1 mM 2,4-DOPA

Triangle = 2.5 mM 2,4-DOPA

Square = 5 mM 2,4-DOPA


10^5 Cells
6
4
2
0
Panel A: Total Cells
0
1
2
3
4
5
6
7
Days of Treatment


10^5 Cells
Panel B: Melanized Cells
2
1
0
0
1
2
3
4
5
6
7
Days of Treatment


10^5 Cells
4
3
2
1
0
Panel C: Non-Melanized Cells
0
1
2
3
4
5
6
7
Days of Treatment

the melanized subpopulation within cultures treated with 1 or 2.5 mM 2,4-DOPA.

A strong cytotoxic effect directed against the melanized cells (Figure 4-5, panel B) was observed on all days for all doses of 2,4-DOPA. In contrast, cytotoxicity directed against the non-melanized cells (Figure 4-5, panel C) was significantly less pronounced ($P < 0.0005$, 1-tailed t-test of % of control, paired by day and dose). Treatment with 5 mM 2,4-DOPA for 7 days resulted in a decrease in the number of melanized cells to 60.9% of the initial number present on day 0, compared to an increase in the number of non-melanized cells to 1.8-times the initial value. 1 and 2.5 mM 2,4-DOPA exhibited similar degrees of cytotoxicity against the melanized cells; the number of melanized cells initially increased to a plateau of 1.8-times the initial number (cf., control cultures which grew to 3-times their initial cell number), then, by day 7, the number began to decrease back towards initial levels. In contrast, the non-melanized cells in cultures treated at 1 and 2.5 mM 2,4-DOPA continued to grow until they reached a greater number than in control cultures. The data indicate that the non-melanized cells had filled in the space vacated by the melanized cells, so that when the whole culture (panel A) was assessed without regard to differential effects upon the two subpopulations, no cytotoxicity was evident at the later time points, despite the significant loss of melanized cells.

Similar trends were evident in a repeat experiment (Table 4-4). For example, after 7 days of treatment with either 1 mM or 2.5 mM 2,4-DOPA, the total number of cells was actually greater in treated

Table 4-4

Cytotoxicity of 2,4-DOPA Against Cloudman Melanoma Cells

Days of Treatment	2,4-DOPA (mM)	10^5 Total Cells (S.D.)	% of Control	10^5 Melanized Cells (S.D.)	% of Control	10^5 Non-Melanized Cells (S.D.)	% of Control
0	0.0	3.20 (0.20)	100.0	1.01 (0.14)	100.0	2.19 (0.18)	100.0
1	0.0	4.01 (0.19)	100.0	2.00 (0.11)	100.0	2.01 (0.11)	100.0
	1.0	3.84 (0.10)	95.8	1.49 (0.12)	74.5	2.35 (0.13) c	116.9
	2.5	3.42 (0.37) a	85.3	1.30 (0.17)	65.0	2.12 (0.25)	105.5
	5.0	2.71 (0.13) b	67.6	0.83 (0.06)	41.5	1.88 (0.10)	93.5
4	0.0	4.18 (0.44)	100.0	2.19 (0.24)	100.0	1.99 (0.22)	100.0
	1.0	4.70 (0.68)	112.4	1.29 (0.23) b	58.9	3.41 (0.51) d	171.4
	2.5	3.27 (0.27) a	78.2	0.90 (0.15) b	41.1	2.37 (0.24)	119.1
	5.0	2.34 (0.19) b	56.0	0.53 (0.08) b	24.2	1.81 (0.16)	91.0
7	0.0	5.27 (0.64)	100.0	2.58 (0.48)	100.0	2.69 (0.49)	100.0
	1.0	7.88 (0.17) d	149.5	2.14 (0.12)	82.9	5.74 (0.17) d	213.4
	2.5	7.32 (0.65) d	138.9	1.93 (0.51) a	74.8	5.39 (0.68) d	200.4
	5.0	3.41 (0.04) b	64.7	0.83 (0.05) b	32.2	2.58 (0.05)	95.9

Triplicate cultures were continuously treated with 2,4-DOPA. Cells were released from monolayers for determination of total cell count. Viability was assessed by exclusion of trypan blue. Cells were also stained in suspension with L-3,4-DOPA for scoring as melanized or non-melanized.

[a] $P < 0.05$, 1-tailed Dunnett's test.

[b] $P < 0.01$, 1-tailed Dunnett's test.

[c] $P < 0.05$, 2-tailed Dunnett's test.

[d] $P < 0.01$, 2-tailed Dunnett's test.

cultures than in control cultures (at 1 mM 2,4-DOPA, 150 % of control; at 2.5 mM 2,4-DOPA, 139 % of control). However, this was due to the continued growth of non-melanized cells in treated cultures. For example, the non-melanized cells in cultures treated for 7 days with 2.5 mM 2,4-DOPA increased in number to 200 % of control. In contrast, the melanized cells decreased in number to 32.2 % of control.

Treatment of cultures with 5 mM 2,4-DOPA almost completely inhibited the increase in total cell number seen in control cultures (cf. day 0 with day 7). The melanized cells decreased in number over the 7-day period, while the non-melanized cells only increased in number to a small extent. In terms of the controls on day 7, the melanized cell number was 32 % of control, and the non-melanized cell number was 96 % of control.

MJY-Alpha Mammary Tumor and L-1210 Leukemia Cells

Continuous treatment of MJY-alpha mammary tumor cells (Table 4-5) or L-1210 leukemia cells (Table 4-6) with 5 mM 2,4-DOPA over a 7-day period resulted in no apparent cytotoxicity as determined by cell count. Alpha cells and L-1210 cells do not contain tyrosinase. The lack of cytotoxicity is in keeping with the absence of tyrosinase, since 2,4-DOPA is a tyrosinase-targeted prodrug.

Further experiments were performed with the alpha cells to test the possibility that 6-hydroxy-DOPA might be generated from 2,4-DOPA independent of tyrosinase, but that the alpha cells may be resistant to 6-hydroxy-DOPA's cytotoxic effects. When cultures were treated overnight with either (1) 20 ug/mL of 6-hydroxy-DOPA (94 uM), or

Table 4-5

Lack of Cytotoxicity of 5 mM 2,4-DOPA Against MJY-Alpha Mammary Tumor Cells

Days of Treatment	10^5 Cells (S.D.)			
	Trial 1		Trial 2	
	Control	Treated	Control	Treated
0	3.69 (0.20)		2.29 (0.11)	
1	8.48 (2.62)	7.46 (0.95)	6.69 (0.45)	6.53 (0.36)
2	9.88 (1.17)	10.08 (0.39)	11.89 (0.58)	10.88 (0.31)
3	12.55 (0.07)	12.30 (0.92)	13.65 (1.85)	14.10 (0.82)
4	16.52 (0.11)	15.88 (1.31)	11.23 (1.17)	12.42 (0.32)
5	15.30 (0.21)	14.88 (0.11)	13.23 (1.45)	14.75 (1.13)
6	15.90 (0.14)	18.18 (1.45)	13.13 (0.67)	13.88 (0.95)
7	17.15 (0.64)	18.70 (1.77)	13.10 (2.40)	13.82 (1.24)

Duplicate cultures were continuously treated with 5 mM 2,4-DOPA for 1-7 days. Viability was assessed by exclusion of trypan blue. There was no significant difference between control and treated cultures in either trial ($P > 0.05$ for both studies, 1-tailed paired t-test of treated versus control).

Table 4-6

Lack of Cytotoxicity of 5 mM 2,4-DOPA Against L-1210 Leukemia Cells

Days of Treatment	10^5 Cells/mL (S.D.)			
	Trial 1		Trial 2	
	Control	Treated	Control	Treated
0	9.75 (0.35)		11.87 (0.81)	
1	8.15 (0.49)	7.50 (0.28)	9.45 (0.28)	9.57 (0.11)
2	8.50 (0.07)	9.45 (0.07)	8.38 (0.25)	8.15 (0.14)
3	10.07 (1.03)	9.35 (0.49)	9.25 (0.07)	8.68 (0.95)
4	8.88 (0.46)	9.20 (0.14)	10.45 (1.06)	9.15 (0.64)
5	8.68 (0.95)	8.90 (0.21)	9.27 (0.46)	9.65 (0.14)
6	9.35 (1.06)	10.17 (2.09)	11.07 (0.04)	10.50 (0.28)
7	10.00 (1.84)	9.90 (1.98)	12.45 (0.28)	11.45 (0.71)

Duplicate cultures were continuously treated with 5 mM 2,4-DOPA for 1-7 days. Viability was assessed by exclusion of trypan blue. There was no significant difference between control and treated cultures in either trial ($P > 0.05$ for both studies, 1-tailed paired t-test of treated versus control).

(2) 1 mM 2,4-DOPA + 0.176 x 10^{-3} units of tyrosinase, or (3) 1 mM 2,4-DOPA + 1.76 x 10^{-3} units of tyrosinase, the number of viable cells decreased from a control value of 13.35 x 10^5 ($\pm$ 0.70 S.D.) to (1) 9.82 x 10^5 ($\pm$ 1.21), (2) 10.70 x 10^5 ($\pm$ 0.47), and (3) 2.33 x 10^5 ($\pm$ 0.60), respectively. All data points were derived from triplicate cultures. As discussed in §3.1.2, the rate of synthesis of 6-hydroxy-DOPA within melanoma cells exposed to 1 mM 2,4-DOPA is expected to be on the order of uM/min. In other studies, it was determined that 1.76 x 10^{-3} units of tyrosinase (i.e., the level used in condition 3, above), in the absence of L-3,4-DOPA as activator, catalyzed the formation of 6-hydroxy-DOPA from 1 mM 2,4-DOPA at a rate of 7.6 uM/hour (Morrison and Cohen 1982). The data above show that significant cytotoxicity against the alpha cells occurs when cultures are subjected to a flux of 6-hydroxy-DOPA approximately 1/60 th of that which would theoretically be generated within melanoma cells during treatment with 1 mM 2,4-DOPA. This demonstrates that the alpha cells are susceptible to 6-hydroxy-DOPA and that they represent an appropriate control for the study of non-tyrosinase mediated effects.

C-1300 Neuroblastoma Cells

Continuous treatment of C-1300 neuroblastoma cultures with 5 mM 2,4-DOPA for 1 day resulted in a small degree of cytotoxicity; the number of viable cells in treated cultures was an average of 79% of control. (The raw data from individual trials are shown in Tables 4-7A and 4-7B). However, the cultures treated at 5 mM were still growing. In trial 1 of Table 4-7A, cultures treated with 5 mM 2,4-

Table 4-7A

Trial of 2,4-DOPA Against C-1300 Neuroblastoma

Day of Treatment	2,4-DOPA (mM)	10^5 Cells (S.D.)	Relative Change in Cell Number After 24 Hours		% of Control
			Control	Treated	
Trial 1					
0	0.0	2.70 (0.46)	----	----	
1	0.0	4.49 (0.28)	1.66		
	1.0	4.50 (0.25)		1.67	100
	2.5	3.89 (0.12)		1.44	87
	5.0	3.85 (0.29)		1.43	86
Trial 2					
0	0.0	1.73 (0.27)	----	----	
1	0.0	3.71 (0.37)	2.14		
	1.0	3.29 (0.35)		1.90	89
	2.5	3.15 (0.17)		1.82	85
	5.0	3.02 (0.36)		1.75	81

Triplicate cultures were continuously treated with 2,4-DOPA for 24 hours. Viability was assessed by exclusion of trypan blue.

Table 4-7B

Effect of Various Enzyme Inhibitors upon 2,4-DOPA-Mediated Cytotoxicity in Neuroblastoma Cultures

Day of Treatment	2,4-DOPA (mM)	Inhibitor[a]	10^5 Cells (S.D.)	Relative Change in Cell Number After 24 Hours		% of Control
				Control (no 2,4-DOPA)	2,4-DOPA	
0	0	----	2.75 (0.25)	----	----	
1	0	----	5.30 (0.61)	1.93		
	5	----	4.03 (0.46)		1.47	76
	0	AMPT	4.97 (0.28)	1.81		
	5	AMPT	4.20 (0.28)		1.53	85
0	0	----	2.63 (0.21)	----	----	
1	0	----	5.97 (0.11)	2.27		
	5	----	4.18 (0.19)		1.59	70
	0	3-I-T	4.81 (0.35)	1.83		
	5	3-I-T	3.47 (0.27)		1.32	72
	0	PTU	5.08 (0.61)	1.93		
	5	PTU	3.73 (0.33)		1.42	73

(continued on next page)

(Table 4-7B, continued)

Day of Treatment	2,4-DOPA (mM)	Inhibitor[a]	10^5 Cells (S.D.)	Relative Change in Cell Number After 24 Hours		% of Control
				Control (no 2,4-DOPA)	2,4-DOPA	
0	0	----	1.72 (0.11)	----	----	
1	0	----	4.24 (0.53)	2.47		
	5	----	3.45 (0.20)		2.01	81
	0	AMPT	4.51 (0.20)	2.62		
	5	AMPT	3.68 (0.10)		2.14	82
	0	3-I-T	4.91 (0.68)	2.85		
	5	3-I-T	3.86 (0.14)		2.24	79
	0	PTU	4.65 (0.24)	2.70		
	5	PTU	3.42 (0.22)		1.99	74

[a]AMPT = alpha-methyl-p-tyrosine; 3-I-T = 3-iodo-p-tyrosine; PTU = phenylthiourea. Triplicate cultures were pretreated with 100 uM inhibitor for 30 minutes before initiation of treatment with 5 mM 2,4-DOPA on Day 0. After 24 hours of treatment, viability was assessed by exclusion of trypan blue.

DOPA increased 1.43-fold in number over the test period while control cultures increased 1.66-fold. In trial 2, cultures treated at 5 mM 2,4-DOPA increased 1.75-fold in number while control cultures increased 2.14-fold. Similar trends are also evident in the data of Table 4-7B.

The cytotoxicity of 5 mM 2,4-DOPA against neuroblastoma cells was less than that against melanized melanoma cells (Table 4-8). One day of treatment with 5 mM 2,4-DOPA slowed the growth rate of neuroblastoma cultures so that the cell number was 79 % of control. In contrast, the melanized cells in treated B-16 and Cloudman melanoma cultures actually decreased in number to an average of 40 % of control. These data indicate that 5 mM 2,4-DOPA exerted 2.9-fold stronger cytotoxicity against melanized melanoma cells than against neuroblastoma cells ($P < 0.0005$, 1-tailed t-test).

A dose response relationship was sought to determine if a dose of 2,4-DOPA could be found which would provide a cytotoxic effect against melanoma cells, but not against neuroblastoma cells. The data of Table 4-9 demonstrate that treatment of neuroblastoma cultures with 1 mM 2,4-DOPA produces a very small effect (94 % of control), which was not statistically significant. However, treatment with 2.5 mM 2,4-DOPA decreased the growth rate of cultures (Table 4-7A) so that the cell number in treated cultures was 86 % of control after one day of treatment (Table 4-9).

The data presented above indicate that a dose of 1 mM 2,4-DOPA may provide differential cytotoxicity against melanoma cells while sparing neuroblastoma cells. Treatment at higher doses of 2,4-DOPA

Table 4-8

Comparison of the Cytotoxicity of 5 mM 2,4-DOPA Against Melanized Melanoma Cells and Neuroblastoma Cells

Cell Type[a]	Source of Data	Relative Change in Cell[b] Number After 24 Hours		% of Control[b]
		Control	Treated 5 mM 2,4-DOPA	
$B\text{-}16_1$	Table 4-2	1.46	0.36	25
	Figure 4-1	0.85	0.08	9
$B\text{-}16_2$	Figure 4-2	1.13	0.49	43
	Table 4-3	0.49	0.25	52
CL	Figure 4-3	2.01	0.87	43
	Table 4-4	1.98	0.82	41
NBL	Table 4-7	1.66	1.43	86
		2.14	1.75	81
	Table 4-9	1.93	1.47	76
		2.27	1.59	70
		2.47	2.01	81

[a]B-16 = B-16 melanoma, melanized cells; CL = Cloudman melanoma, melanized cells; NBL = neuroblastoma.

[b]Mean for each experiment. When individual data points from all experiments were pooled, the % of control $\pm$ S.D. (N) was 78.9 % $\pm$ 8.1 (15) for NBL cells and 39.8 % $\pm$ 17.0 (17) for melanized melanoma cells. (Note: The B-16 and CL data were not significantly different and were, therefore, pooled; $0.2 < P < 0.5$). The neuroblastoma cells were 2.9-fold more senstitive to 2,4-DOPA than the melanized melanoma cells ($P < 0.0005$, 1-tailed t-test).

Table 4-9

Summary of the Effect of 2,4-DOPA Treatment upon Neuroblastoma Cultures

2,4-DOPA (mM)	Viable Cells % of Control ± S.D. (N)
0	100.0 ± 6.7 (15)
1	[a] 94.4 ± 9.4 (6)
2.5	[b] 85.7 ± 3.4 (6)
5	[b] 78.9 ± 8.1 (15)

Cultures were continuously treated with 2,4-DOPA for 24 hours. Viability was assessed by exclusion of trypan blue. Individual data points from the experiments of Tables 4-7A and 4-7B were pooled and normalized to obtain the above %'s.

[a] $P > 0.05$, 1-tailed Dunnett's test.

[b] $P < 0.01$, 1-tailed Dunnett's test.

may entail modest cytotoxicity against neuroblastoma cells, while resulting in substantial cytotoxicity against melanoma cells.

Tyrosine hydroxylase normally converts L-p-tyrosine to L-3,4-DOPA in catecholamine neurons. Since 2,4-DOPA is an analog of L-p-tyrosine, it is theoretically possible that tyrosine hydroxylase, although acting via a different reaction mechanism than tyrosinase, may also convert 2,4-DOPA to 6-hydroxy-DOPA in neuroblastoma cells. In an effort to determine if the cytotoxic effect of 2,4-DOPA upon neuroblastoma cells was due to generation of 6-hydroxy-DOPA by tyrosine hydroxylase, cultures were pretreated for 30 minutes with 100 uM alpha-methyl-p-tyrosine (AMPT, K_I for tyrosine hydroxylase = 17 uM (Udenfriend et al. 1965)) or 100 uM 3-iodo-tyrosine (K_I = 0.4 uM (Udenfriend et al. 1965)) then treated with 5 mM 2,4-DOPA in the presence of AMPT or 3-iodo-tyrosine. For comparison, cultures which were not exposed to AMPT or 3-iodo-tyrosine were treated with 2,4-DOPA alone. Controls were exposed to medium with or without inhibitor. In addition, 100 uM phenylthiourea (PTU), an inhibitor of tyrosinase (Lerner and Fitzpatrick 1950), was also tested in order to rule the possibility that the cytotoxicity of 2,4-DOPA against neuroblastoma cells was due to tyrosinase (note: 100 uM PTU was sufficient to block the cytotoxicity of 2,4-DOPA against the B-16 cells; see §4.1.2). The data shown in Table 4-10 indicate that neither 100 uM AMPT, 100 uM 3-iodo-tyrosine, nor 100 uM PTU blocked the cytotoxic effect of 2,4-DOPA upon neuroblastoma cells. The mean effect of 2,4-DOPA in the presence of the various enzyme inhibitors was 79 % of control for 2,4-DOPA alone, 83 % of control for 2,4-DOPA + AMPT, 75 % of control

Table 4-10

Summary of the Effect of Various Enzyme Inhibitors upon 2,4-DOPA-Mediated Cytotoxicity in Neuroblastoma Cultures

2,4-DOPA (mM)	Inhibitor[a] 100 uM	Viable Cells % of Control ± S.D. (N)
5	----	78.9 ± 8.1 (15)
5	AMPT	83.0 ± 8.8 (6)
5	3-I-T	75.4 ± 5.4 (6)
5	PTU	73.5 ± 5.1 (6)

[a]AMPT = alpha-methyl-p-tyrosine; 3-I-T = 3-iodotyrosine; PTU = phenylthiourea. Cultures were pretreated with inhibitor for 30 minutes before initiation of treatment with 2,4-DOPA. After 24 hours of treatment, viability was assessed by exclusion of trypan blue. Individual data points from the experiments of Tables 4-7A and 4-7B were pooled and normalized to obtain the above %'s. All $P > 0.05$, 1-tailed Dunnett's test.

for 2,4-DOPA + 3-I-tyrosine, and 74 % of control for 2,4-DOPA + PTU. None of the groups receiving enzyme inhibitors exhibited significantly less cytotoxicity than the group which received 2,4-DOPA alone (all $P > 0.05$, 1-tailed Dunnett's test). Therefore, it appears that the cytotoxicity of 2,4-DOPA against C-1300 neuroblastoma cells was non-specific and was not due to either tyrosine hydroxylase or tyrosinase.

4.1.2: Blockade of 2,4-DOPA-Mediated Cytotoxicity by Phenylthiourea, an Inhibitor of Tyrosinase

The role of tyrosinase as the cellular activator of the prodrug, 2,4-DOPA, was evaluated by addition of the copper-binding agent phenylthiourea (PTU), an inhibitor of tyrosinase (Lerner and Fitzpatrick 1950). The results of three trials are shown in Table 4-11A. Phenylthiourea at either 50 uM or 100 uM suppressed the cytotoxicity of 2,4-DOPA. Pooled results from days 1 and 2 in Table 4-11A and Table 4-11B are presented in Table 4-12. The cytotoxicity of 2,4-DOPA varied with the concentration of PTU: in the absence of PTU, the total cell number in treated cultures was 54 % of control; in the presence of 50 uM PTU, 75 % of control; and in the presence of 100 uM PTU, 90 % of control. The suppression of 2,4-DOPA-mediated cytotoxicity was significant at both concentrations of PTU ($P < 0.01$, 1-tailed Dunnett's test). These results verify that the toxicity of 2,4-DOPA against melanoma cells is mediated by tyrosinase.

The protective effect of PTU was examined further, since it was possible that PTU, like other thiol compounds, could be an effective free radical scavenger. Furthermore, it was necessary to control for

Table 4-11A

Suppression of 2,4-DOPA-Mediated Cytotoxicity Against B-16[2] Melanoma by Phenylthiourea (PTU), an Inhibitor of Tyrosinase

	2,4-DOPA (mM)	PTU (uM)	Day 1 10^5 Cells(S.D.)	Day 1 % of Control	Day 2 10^5 Cells(S.D.)	Day 2 % of Control
a	0	0	3.44 (0.47)	100.0	4.22 (0.61)	100.0
	5		2.38 (0.26)	69.2	2.19 (0.14)	51.9
	0	50	3.30 (0.25)	100.0	3.37 (0.73)	100.0
	5		2.88 (0.25)	87.3	2.46 (0.38)	73.0
	0	100	3.13 (0.17)	100.0	3.38 (0.33)	100.0
	5		3.02 (0.25)	96.5	3.46 (0.37)	102.4
b	0	0	3.89 (0.44)	100.0	4.72 (0.02)	100.0
	5		1.55 (0.28)	39.8	1.48 (0.18)	31.4
	0	50	3.41 (0.11)	100.0	3.15 (0.24)	100.0
	5		2.33 (0.00)	68.3	2.15 (0.07)	68.3
	0	100	2.54 (0.20)	100.0	3.42 (0.19)	100.0
	5		2.35 (0.42)	92.5	2.23 (0.35)	65.2
c	0	0	2.77 (0.02)	100.0		
	5		1.39 (0.28)	50.2		
	0	100	2.08 (0.18)	100.0		
	5		2.07 (0.08)	99.5		

Cultures were preincubated for either 30 minutes (a and b) or 24 hours (c) with the appropriate concentration of PTU before initiation of treatment with 2,4-DOPA plus PTU on Day 0. Parallel experiments were performed in the absense of PTU. Cell counts were performed in triplicate in trial "a" and in duplicate in trials "b" and "c" and represent total cells (i.e., both melanized and non-melanized). Viability was assessed by exclusion of trypan blue.

[a]1.14 ($\pm$ 0.09 S.D., N=3) x 10^5 cells were present on Day 0.

[b]1.13 ($\pm$ 0.15 S.D., N=2) x 10^5 cells were present on Day 0.

[c]1.35 ($\pm$ 0.00 S.D., N=2) x 10^5 cells were present in cultures pretreated for one day with medium alone. 1.41 ($\pm$ 0.08 S.D., N=2) x 10^5 cells were present in cultures pretreated for one day with PTU.

Table 4-11B

Suppression of 2,4-DOPA-Mediated Cytotoxicity Against B-16$_2$ Melanoma by Phenylthiourea (PTU); Lack of Protective Effect of Control Compounds: Phenylurea (PU) and Thiourea (TU)

	2,4-DOPA (mM)	PTU (uM)	PU (uM)	TU (uM)	10^5 Cells (S.D.)	% of Control
a	0	-	-	-	2.81 (0.21)	100.0
	5	-	-	-	1.66 (0.09)	59.1
	0	100	-	-	2.82 (0.11)	100.0
	5	100	-	-	2.15 (0.25)	76.2
	0	-	100	-	2.56 (0.37)	100.0
	5	-	100	-	1.37 (0.04)	53.5
	0	-	-	100	2.49 (0.09)	100.0
	5	-	-	100	1.55 (0.26)	62.2
b	0	-	-	-	1.32 (0.13)	100.0
	5	-	-	-	0.82 (0.14)	62.1
	0	100	-	-	1.27 (0.14)	100.0
	5	100	-	-	1.18 (0.09)	92.9
	0	-	100	-	1.59 (0.09)	100.0
	5	-	100	-	1.02 (0.11)	64.2
	0	-	-	100	1.51 (0.21)	100.0
	5	-	-	100	0.64 (0.10)	42.4

Triplicate cultures were preincubated for 30 minutes with either PTU, PU, or TU before the initiation of treatment. Cultures were treated for 24 hours with 2,4-DOPA $\pm$ PTU, PU, or TU. Parallel experiments were performed in the absense of PTU, PU, or TU. Cell counts represent total cells (i.e., both melanized and non-melanized). Viability was assessed by exclusion of trypan blue.

[a] 2.30 ($\pm$ 0.28 S.D., N=3) x 10^5 cells were present on Day 0.

[b] 0.65 ($\pm$ 0.04 S.D., N=3) x 10^5 cells were present on Day 0.

Table 4-12

Summary of the Effect of PTU upon 2,4-DOPA-Mediated Cytotoxicity in $B\text{-}16_2$ Melanoma Cultures

2,4-DOPA (mM)	PTU[a] (uM)	Viable Cells % of Control ± S.D. (N)
5	0	54.0 ± 13.3 (18)
5	50	[b] 75.4 ± 10.6 (10)
5	100	[b] 89.8 ± 14.6 (18)

[a]PTU = phenylthiourea. Cultures were pretreated with inhibitor before initiation of treatment with 2,4-DOPA. After 24 hours of treatment, viability was assessed by exclusion of trypan blue. Individual data points from the experiments of Tables 4-11A and 4-11B were pooled and normalized to obtain the above %'s.

[b]$P < 0.01$, 1-tailed Dunnett's test.

the possibility that PTU may block cytotoxicity by inhibiting transport of 2,4-DOPA across the cell membrane. Controls were run utilizing compounds which varied from PTU in the critical properties of interest. As a control for free radical scavenging, thiourea was substituted for PTU. Thiourea is an effective scavenger of free radicals (k for reaction with $^{\cdot}OH = 4.7 \times 10^{9}$, Anbar and Neta 1967) but a poor inhibitor of tyrosinase (1.5 - 2 mM thiourea is needed to inhibit the dopa oxidase reaction of mammalian tyrosinase by 50%, Pomerantz 1963). As a control for possible interference with 2,4-DOPA transport by the phenyl ring of PTU, phenylurea was substituted for PTU. Treated cultures were incubated with 5 mM 2,4-DOPA in either medium alone or medium plus either 100 uM PTU, phenylurea, or thiourea; controls were incubated in medium with or without inhibitor. The results (Table 4-11B) demonstrate that neither 100 uM phenylurea nor thiourea provided protection against 2,4-DOPA-mediated cytotoxicity whereas 100 uM PTU did suppress cytotoxicity. When the individual data points from trials in Tables 4-11A and 4-11B were pooled (Table 4-13), the number of viable cells in treated cultures was 54 % of control for treatment with 2,4-DOPA alone, 59 % of control for treatment with 2,4-DOPA plus phenylurea, 52 % of control for treatment with 2,4-DOPA plus thiourea, and 90 % of control for treatment with 2,4-DOPA plus PTU ($P > 0.05$ for phenylurea and thiourea, $P < 0.01$ for PTU; 1-tailed Dunnett's test). The above results indicate that the thiourea and phenylurea moieties of the PTU molecule are not sufficient by themselves to block 2,4-DOPA-mediated cytotoxicity against melanoma cells. The entire PTU molecule is

Table 4-13

Summary of the Effect of PTU and PTU Analogs upon 2,4-DOPA-Mediated Cytotoxicity in B-16$_2$ Melanoma Cultures

2,4-DOPA (mM)	Inhibitor[a] 100 uM		Viable Cells % of Control ± S.D. (N)
5	----		54.0 ± 13.3 (18)
5	PTU	b	89.8 ± 14.6 (18)
5	PU	c	58.8 ± 7.1 (6)
5	TU	c	52.4 ± 13.4 (6)

[a]PTU = phenylthiourea; PU = phenylurea; TU = thiourea. Cultures were pretreated with inhibitor before initiation of treatment with 2,4-DOPA. After 24 hours of treatment, viability was assessed by exclusion of trypan blue. Individual data points from the experiments of Tables 4-11A and 4-11B were pooled and normalized to obtain the above %'s.

[b]$P < 0.01$, 1-tailed Dunnett's test.

[c]$P > 0.05$, 1-tailed dunnett's test.

required, in keeping with its known inhibitory action upon tyrosinase.

4.1.3: Effect of 2,4-DOPA upon DNA, RNA, and Protein Synthesis

The effect of 2,4-DOPA treatment upon macromolecular synthesis was evaluated in cultures of B-16_2 melanoma. To study the effects of treatment with 2,4-DOPA upon DNA, RNA, and protein synthesis, labeled precursors were added to cultures which had been preincubated for 3 hours with 5 mM 2,4-DOPA in growth medium. After 1 hour of further incubation with 2,4-DOPA and label, the cultures were processed to measure incorporation of label into TCA-insoluble material. Controls were preincubated with media alone before labeling.

The data presented in Table 4-14 (section A) demonstrate that DNA, RNA, and protein synthesis were depressed in a dose-dependent manner by 4 hours of treatment with 2,4-DOPA. For example, at a dose of 5 mM 2,4-DOPA, DNA synthesis was 20.6% of control, RNA synthesis was 44.4% of control, and protein synthesis was 24.6% of control. Parallel cultures which were incubated with either medium or medium plus 5 mM 2,4-DOPA were released with trypsin/EDTA, and the number of viable cells counted. In 3 of the 4 experiments which are pooled in Table 4-14, the number of viable cells was determined 5 hours after initiation of treatment (i.e., these cultures were treated for 1 hour longer than the cultures used in the labeling experiment). The mean ratio of cell number in treated versus control cultures was 1.02, 1.17, and 1.06. Since the number of viable cells was not less in treated cultures compared to control, the generalized decrease in macromolecular synthesis was not due to simply a decrease in the

Table 4-14

Treatment of B-16[2] Melanoma Cultures with 2,4-DOPA: Effect upon DNA, RNA, and Protein Synthesis

2,4-DOPA (mM)	DNA % of Control (S.D.) N	RNA % of Control (S.D.) N	Protein % of Control (S.D.) N
A) 3 Hours Treatment with 2,4-DOPA Followed by 1 Hour Treatment with 2,4-DOPA and Label			
0.0	100.0 (3.3) 8	100.0 (1.1) 8	100.0 (1.6) 8
1.0	69.1 (10.9) 4	85.1 (7.3) 4	79.1 (4.9) 4
2.5	39.4 (9.3) 4	62.8 (12.9) 4	40.9 (6.6) 4
5.0	20.6 (4.4) 8	44.4 (7.1) 8	24.6 (3.4) 8
B) 1 Hour Treatment with 2,4-DOPA and Label			
0.0	100.0 (2.0) 4	100.0 (3.2) 4	100.0 (2.1) 4
5.0	63.5 (11.2) 4	70.5 (6.4) 4	93.3 (3.2) 4

Each point represents pooled data from either 2 or 4 experiments with an N of 2 for each separate experiment. In section A, all $P < 0.01$ for treated cultures (1-tailed Dunnett's test). In section B, $P < 0.0005$ for DNA and RNA synthesis, and $0.005 < P < 0.01$ for protein synthesis (1-tailed t-test).

number of viable cells, but was due to the effect of 2,4-DOPA upon viable cells. In the fourth experiment, viable cell counts were delayed. After 4.5 hours of treatment, the medium was replaced with fresh medium without drug. The number of viable cells was then determined 5 hours later (mean ratio of treated/control = 0.89).

Two types of controls were run for the above experiments. It was reasoned that since the cytotoxicity of 2,4-DOPA against melanoma cells is dependent upon tyrosinase activity, that the following should be true: (1) Cells lacking tyrosinase should not be affected. (2) The effect upon melanoma cells should be time dependent, and as more 6-hydroxy-DOPA is generated, the degree of cytotoxicity should increase.

The time dependence of depression of macromolecular synthesis by 2,4-DOPA was examined by simultaneously adding both isotopic precursor and 2,4-DOPA to B-16_2 cultures and incubating for 1 hour before processing (Table 4-14, section B). Controls received isotopic precursor only. Comparison of the 4-hour (A) and 1-hour (B) treatment data in Table 4-14 demonstrates that the effect of 2,4-DOPA upon macromolecular synthesis was time-dependent. For example, 4 hours of treatment with 5 mM 2,4-DOPA decreased DNA synthesis to 20.6% of control, whereas DNA synthesis was 63.5% of control after 1 hour of treatment. The effect of 2,4-DOPA upon RNA and protein synthesis was also observed to be less at 1 hour compared to 4 hours of treatment (Table 4-14).

MJY-alpha cells, which do not contain tyrosinase, were treated for 3 hours with 5 mM 2,4-DOPA and labeled for an additional hour in

Table 4-15

Treatment of MJY-Alpha Mammary Tumor Cultures with 2,4-DOPA: Effect upon DNA, RNA, and Protein Synthesis

2,4-DOPA (mM)	DNA % of Control (S.D.) N	RNA % of Control (S.D.) N	Protein % of Control (S.D.) N
0	100.0 (6.4) 4	100.0 (2.9) 4	100.0 (1.3) 4
5	a 95.9 (2.5) 4	a 98.0 (1.8) 4	b 98.3 (7.4) 4

Treated cultures were incubated for 3 hours with 2,4-DOPA, then for an additional hour with 2,4-DOPA plus label. Control cultures were incubated with medium for 3 hours, then for an additional hour with label. The results are pooled from 2 experiments.

[a] $0.1 < P < 0.25$, 1-tailed t-test.

[b] $P > 0.25$, 1-tailed t-test.

the presence of 2,4-DOPA as described above. As shown in Table 4-15, 2,4-DOPA had no effect upon macromolecular synthesis in these cultures. This result is in keeping with the lack of cytotoxicity 2,4-DOPA against MJY-alpha mammary tumor cells (Table 4-5).

4.1.4: Effect of 2,4-DOPA upon Viability, Number of Melanized Cells per Culture, Tyrosinase Activity, and Content of Prelabeled Melanin

The effect of 2,4-DOPA treatment upon melanized B-16_2 melanoma cells was studied in the following experiments in which the number of melanized cells, tyrosinase activity, and the disposition of intracellular prelabeled melanin were monitored. Melanin was prelabeled by incubation of cultures with (^{14}C)-D,L-DOPA. After incubation in medium for 2 days to allow efflux of unincorporated label, cultures were treated for 1 day with 5 mM 2,4-DOPA, and then the radioactivity released into the supernatant and that retained by the cell monolayers were quantitated. The numbers of viable cells and the distribution of cells into melanized and non-melanized subpopulations were determined in replicate control and treated cultures. Tyrosinase activity was measured by the method of Wick et al. (1979b).

In trial 1, the total number of viable cells decreased to 82% of control after 1 day of treatment with 5 mM 2,4-DOPA, and the melanized subpopulation decreased to 29% of control (Table 4-16). The tyrosinase activity decreased to 25% of control following 1 day of treatment, thereby confirming the results of melanized cell counts. In trial 2, the total number of viable cells decreased to 64 % of

Table 4-16

Treatment of B-16$_2$ Melanoma Cultures with 2,4-DOPA: Effect upon Survival of Total and Melanized Cells, Tyrosinase Activity, and Content of Prelabeled Melanin Within the Monolayer

	2,4-DOPA (mM)	10^5 Total Cells (S.D.)	% of Control	10^5 Melanized Cells (S.D.)	% of Control	Tyrosinase DPM/Culture (S.D.)	% of Control	DPM Remaining In Monolayer (% of Total)[c]
a	0	4.69 (0.93)		1.62 (0.36)		7765 (333)		92.54 (0.49)
	5	3.85 (0.64)	82.1	0.47 (0.12)	29.0	1937 (5)	24.9	85.24 (0.45)
b	0	3.30 (0.57)		1.05 (0.18)		18670 (1972)		97.10 (0.08)
	5	2.10 (0.27)	63.6	0.46 (0.07)	43.8	8356 (757)	44.8	89.29 (1.64)

Melanoma cultures were treated with 5 mM 2,4-DOPA. Control cultures were incubated in medium. After 24 hours of treatment, viablility was assessed by exclusion of trypan blue. Cells were also stained with L-3,4-DOPA to enhance the melanin content of melanized cells prior to scoring cells as melanized or non-melanized. Tyrosinase activity was measured by the method of Wick et al. (1979b). Parallel cultures were labeled with (C-14)-D,L-DOPA 2 days prior to treatment in order to label melanin. The number of dpm in both the supernatant and the monolayer of treated and control cultures was determined.

[a] N=2 for all points.

[b] N=4 for all points. In trial b, the cell count was 2.95 ($\pm$ 0.29 S.D., N=4) x 10^5 on day 0.

[c] Total dpm = dpm(supernatant) + dpm(monolayer)

control. The number of melanized cells decreased to 44 % of control, and the tyrosinase activity decreased to 45 % of control.

The total number of dpm in each culture (supernatant + monolayer) was determined and used to calculate the percentage of the total counts which remained in the monolayer. In both trials, treatment of cultures with 2,4-DOPA resulted in a decrease in dpm in the monolayer (7.3% decrease in trial 1 and 7.8% in trial 2; Table 4-16). The decrease was significant in both trials ($0.001 < P < 0.0025$ for trial 1 and $P < 0.0005$ for trial 2, 1-tailed t-test). However, the decrease in dpm was less than would be expected if 2,4-DOPA treatment resulted in lysis of melanized cells and spillage of labeled melanin into the medium. The most likely explanation for this result is that fragments of lysed cells containing labeled melanin may remain attached to the monolayer thereby resulting in a larger retention of label by the monolayer than would be expected by viable cell count. This hypothesis was tested in the following experiment. $B\text{-}16_2$ melanoma cultures were labeled with (^{14}C)-D,L-DOPA as described above, but instead of treating with 2,4-DOPA, cultures were exposed to distilled water for 5 minutes. Controls were incubated in Hank's balanced salt solution. Immediately following incubation, cultures were gently washed 3-times with balanced salt solution, dissolved overnight in 1 N KOH, and the amount of label retained by the monolayer was quantitated. Parallel cultures were incubated in either water or balanced salt solution for 5 minutes, washed 3-times in balanced salt solution, and released with trypsin/EDTA for cell count. Viability was assessed by exclusion of trypan blue. Incubation for 5 minutes in

Table 4-17

Incubation of B-16_2 Melanoma Cultures in Water: Retention of Prelabeled Melanin

	10^5 Viable Cells (S.D.)	% of Control	D.P.M. in Monolayer (S.D.)	% of Control
Control	6.26 (0.14)	100.0	157,946 (10,480)	100.0
Water	0.68 (0.59)	10.9	142,334 (15,016)	92.5

Cultures were incubated for 5 minutes in distilled water. Control cultures were incubated in Hank's balanced salt solution. Cultures were then washed 3 times with balanced salt solution and viability assessed by exclusion of trypan blue. Parallel cultures which had been prelabeled with (C-14)-D,L-DOPA (labels melanin) 2 days prior to the experiment were also incubated for 5 minutes in either water or balanced salt solution. The cultures were then washed 3 times in balanced salt solution and dissolved overnight in 1 N KOH. An aliquot of the KOH solution was counted in Liquiscint. All points represent the mean of triplicate cultures.

water decreased the number of viable cells to 11 % of control, while the number of dpm retained by the monolayer was 90 % of control (Table 4-17). These results indicate that the apparent failure of 2,4-DOPA to decrease the amount of prelabeled melanin in the monolayer was most probably due to retention of cell fragments on the monolayer. Of interest is the fact that the decrease in label in the monolayer brought about by either 2,4-DOPA or water treatment was of the same order of magnitude (i.e., 7.6 % decrease versus 9.9 % decrease). This indicates that the decrease in labeled melanin in the monolayer following treatment with 2,4-DOPA approached that which would be theoretically predicted by cell death.

4.1.5: Inhibition of Tyrosine Transport by 2,4-DOPA

Experiments were conducted to determine if 2,4-DOPA, an analog of tyrosine, is transported via the same route as tyrosine. If 2,4-DOPA and tyrosine utilize a common means of transport across the plasma membrane, then 2,4-DOPA would compete for the carrier and decrease the amount of tyrosine transported into the cell. The data shown in Figures 4-6 and 4-7 demonstrate that 2,4-DOPA does compete with tyrosine for entry into the cell. For example, at 30 seconds, the amount of tyrosine transported into B-16_2 cells was 77% of control in the presence of 25 uM 2,4-DOPA and 48% of control in the presence of 75 uM 2,4-DOPA. Tyrosine was present extracellularly at 150 nM. A similar effect was seen with MJY-alpha cultures. For example, at 30 seconds, the amount of tyrosine transported was 69% of control in the presence of 75 uM 2,4-DOPA.

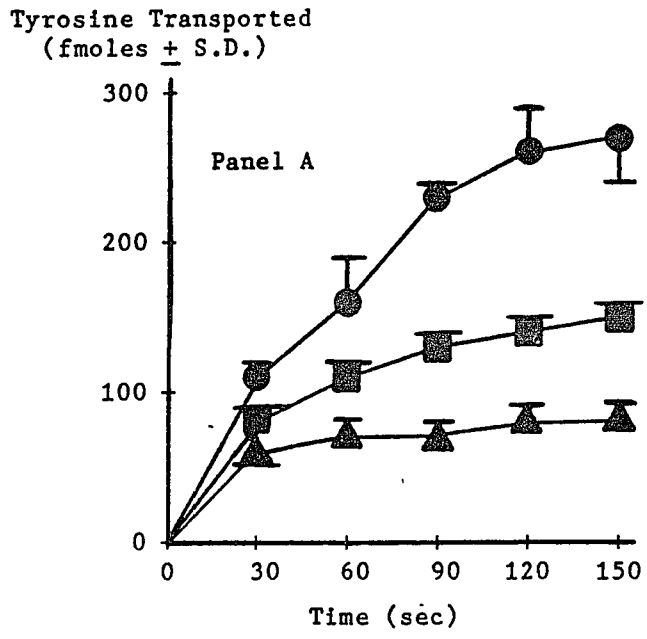
Figure 4-6. 2,4-DOPA-Mediated Inhibition of Tyrosine Transport into $B\text{-}16_2$ Melanoma Cells

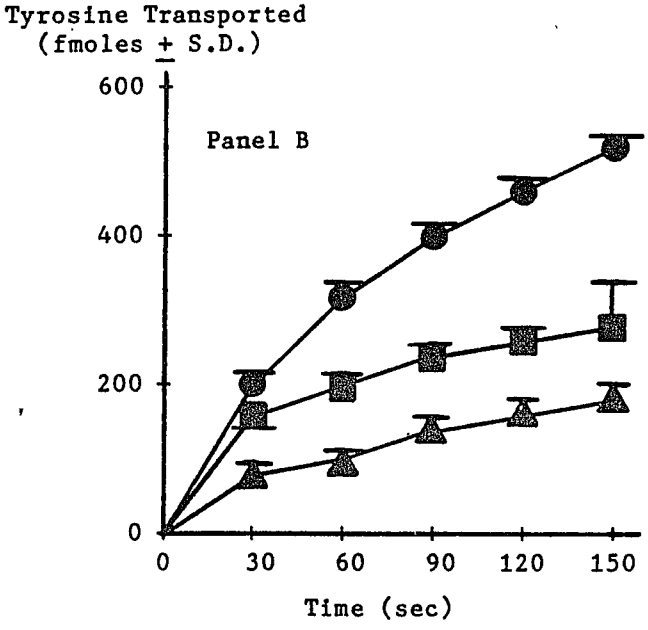
$B\text{-}16_2$ melanoma cultures were first incubated for 30 minutes in balanced salt solution to deplete intracellular amino acid pools. The cultures were then incubated in balanced salt solution containing 2,4-DOPA (25 or 75 uM) and tritiated tyrosine (150 nM). Controls were incubated with tritiated tyrosine alone. At 30 second intervals, cultures were washed and dissolved in 1 N KOH for counting. The amount of tritiated tyrosine transported into the cultures was calculated from the d.p.m./culture and the specific activity of the tritiated tyrosine (33.4 Curies/mmole). All points represent parallel triplicate experiments. In panel A, there were 0.58×10^5 ($\pm$ 0.04 S.D., N=3) cells/culture and in panel B, 0.76×10^5 ($\pm$ 0.05 S.D., N=3) cells/culture.

Circle = Control

Square = 25 uM 2,4-DOPA

Triangle = 75 uM 2,4-DOPA


Tyrosine Transported
(fmoles ± S.D.)
300
200
100
0
Panel A
0
30
60
90
120
150
Time (sec)


Tyrosine Transported
(fmoles ± S.D.)
600
400
200
0
Panel B
0
30
60
90
120
150
Time (sec)

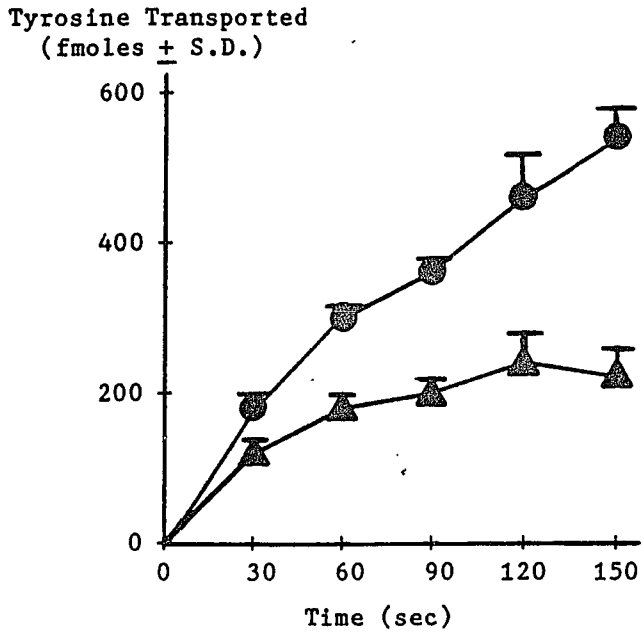


Figure 4-7. 2,4-DOPA-Mediated Inhibition of Tyrosine Transport into MJY-Alpha Mammary Tumor Cells

MJY-alpha mammary tumor cultures were first incubated for 30 minutes in balanced salt solution to deplete intracellular amino acid pools. The cultures were then incubated in balanced salt solution containing 75 uM 2,4-DOPA and 150 nM tritiated tyrosine. Controls were incubated with tritiated tyrosine alone. At 30 second intervals, cultures were washed and dissolved in 1 N KOH for counting. The amount of tritiated tyrosine transported into the cultures was calculated from the d.p.m./culture and the specific activity of the tritiated tyrosine (33.4 Curies/mmole). All points represent parallel triplicate experiments. There were 1.60×10^5 (± 0.17 S.D., N=3) cells/culture.

Circle = control

Triangle = 75 uM 2,4-DOPA

As a control for the above experiments, it was endeavored to determine if the (^{3}H)-tyrosine was actually entering the cell when 2,4-DOPA was present. To this end, the amount of TCA-precipitable radioactivity (i.e., melanin and protein) was determined in B-16$_2$ melanoma cultures incubated for 9 minutes, washed, precipitated with 10% TCA at 11 minutes, and washed again. It was found that in the presence of 75 uM 2,4-DOPA, the number of TCA-precipitable counts was 85.6 % of control ($\pm$ 1.5 S.D., N=4). Therefore, it was concluded that although tyrosine transport was impeded, tyrosine did enter the cell and the steady-state levels of tyrosine were sufficient to support melanin and/or protein synthesis.

4.2: DISCUSSION OF CELL CULTURE RESULTS

4.2.1: Experiments with Melanoma Cultures

B-16 and Cloudman melanoma cultures were used as an in vitro model for human melanoma. When B-16 melanoma cells were grown in vivo, virtually all of the cells contained melanin pigment (Figure 4-1, panel A). However, when carried in culture, the degree of melanization visibly decreased and the cultures consisted of a mixture of melanized and non-melanized cells (Figure 4-1, panel B). The Cloudman cultures were also a mixed population of melanized and non-melanized cells (Figure 4-4, panel A). When melanoma cells are rapidly growing, the tyrosinase activity is greatly suppressed. Once the cultures have reached saturation density, the tyrosinase activity then peaks (§1.7) and the cultures become visibly more melanized. Perhaps the conditions of cell culture, coupled to the possibility that unknown inducers of melanization may be present in vivo but missing in culture, contributes to the loss of melanization in vitro. Since the melanized cell is the predominant cell type seen in vivo, the melanized cells within the cultures were monitored to provide an in vitro model which would be closer to the in vivo state.

2,4-DOPA was demonstrated to be cytotoxic to melanoma cultures over the tested range of 1-5 mM. The following observations support the hypothesis that the cytotoxicity of 2,4-DOPA was mediated by tyrosinase: (1) PTU, a specific inhibitor of tyrosinase, blocked 2,4-DOPA-mediated cytotoxicity. (2) 2,4-DOPA produced a greater degree of cytotoxicity against the melanized cells than against the non-

melanized cells. (3) Treatment of melanoma cultures with 2,4-DOPA resulted in a decrease in tyrosinase activity which paralleled the decrease in melanized cell number. (4) Other cell types which do not contain tyrosinase were either not affected by treatment with 5 mM 2,4-DOPA (MJY-alpha mammary tumor and L-1210 leukemia cells) or affected to a degree 2.9-fold less than melanized melanoma cells (C-1300 neuroblastoma cells).

Since the non-melanized melanoma cells did not contain histochemically demonstrable tyrosinase activity, they served as an internal control in the experiments. Although cytotoxicity was not expected against the non-melanized cells, two likely mechanisms could explain the observed cytotoxicity: (1) Tyrosinase might be released into the medium following cytolysis of melanized cells, thereby generating 6-hydroxy-DOPA extracellularly. (2) 6-hydroxy-DOPA might diffuse out of melanized cells and directly affect neighboring non-melanized cells. In this regard, it is of interest that melanin intermediates are able to diffuse out of melanoma cells to exert cytotoxic effects upon co-cultured fibroblasts (Halaban and Lerner 1977b).

The finding of cytotoxicity against the non-melanized cells is not a drawback of 2,4-DOPA treatment, but actually an advantage. Since 2,4-DOPA was not 100% effective against the melanized cells in vitro, it is apparent that a subpopulation of melanized cells may be resistant. In an in vivo setting, these cells may succumb over long-term treatment to the combined cytotoxic effect of intracellularly generated 6-hydroxy-DOPA plus extracellular 6-hydroxy-DOPA generated

by neighboring cells.

Although possible, it is unlikely that extracellular 6-hydroxy-DOPA within the tumor would cause systemic neurotoxicity. One reason is that it would be diluted many-fold into the venous circulation before gaining access to catecholamine neurons. For example, dilution of the contents of a 1 cm^3 tumor into the total blood volume (5 liters in humans) represents a 0.02% dilution. Furthermore, it has been demonstrated that a prerequisite for the production of neurotoxicity by 6-hydroxy-DOPA is prior extraneuronal decarboxylation to form 6-hydroxydopamine which subsequently is taken up by catecholamine neurons. Pretreatment with either decarboxylase inhibitors or inhibitors of the amine uptake pump blocks neurotoxicity (Kostrzewa and Jacobowitz 1974). Apparently, decarboxylation to 6-hydroxydopamine allows the toxin to be selectively accumulated and concentrated in catecholamine neurons, whereas 6-hydroxy-DOPA, being an amino acid, would be distributed to all tissues, and its effective concentration reduced accordingly. Therefore, if systemic toxicity should prove to be a problem in future in vivo studies, co-administration of 2,4-DOPA with carbidopa, a decarboxylase inhibitor, and mazindol, an inhibitor of the neuronal amine uptake mechanism, may provide protection of catecholamine neurons.

The effect of 2,4-DOPA treatment upon macromolecular synthesis was also examined. It was found that 2,4-DOPA decreased the synthesis of DNA, RNA, and protein. These results are consistent with the production of a general metabolic insult such as would be caused by the production of toxic oxy-radicals by tyrosinase-generated 6-

hydroxy-DOPA. The inhibition was also time-dependent, consistent with the hypothesis that the prodrug, 2,4-DOPA, is hydroxylated by tyrosinase to form 6-hydroxy-DOPA.

2,4-DOPA was demonstrated to block the transport of tyrosine into $B\text{-}16_2$ melanoma cells. This observation suggests that one means by which 2,4-DOPA may enter cells is via the amino acid transport system present in the plasma membrane.

4.2.2: Experiments with Cell Types Which Do Not Contain Tyrosinase

It was found that treatment with 5 mM 2,4-DOPA for 7 days did not produce cytotoxicity against either MJY-alpha cells or L-1210 leukemia cells, as monitored by viable cell counts. This was an expected result, since neither cell type contains tyrosinase.

Experiments were run to evaluate the susceptibility of MJY-alpha cells to 6-hydroxy-DOPA as a control for the possibility that (a) 6-hydroxy-DOPA might be generated from 2,4-DOPA by another route, but that (b) MJY-alpha cells might be resistant to 6-hydroxy-DOPA's cytotoxic effects. MJY-Alpha cells were shown to be susceptible to added 6-hydroxy-DOPA (94 uM) or to added 2,4-DOPA plus tyrosinase. The concentrations of tyrosinase and 2,4-DOPA were chosen so that the flux of 6-hydroxy-DOPA would be in the uM/hour range. As discussed in §3.1.2, the expected rate of synthesis of 6-hydroxy-DOPA from 2,4-DOPA within melanoma tumors is in the uM/minute range. Therefore, the MJY-alpha cells were susceptible to fluxes of 6-hydroxy-DOPA much less than that expected to be generated within melanoma tissue. The data indicate that the resistance of the MJY-alpha cells to 2,4-DOPA was

due to lack of conversion of 2,4-DOPA to 6-hydroxy-DOPA within MJY-alpha cells.

The effect of 2,4-DOPA upon macromolecular synthesis in cultures of MJY-alpha cells was evaluated. It was found that treatment with 5 mM 2,4-DOPA for 4 hours had no effect upon DNA, RNA, or protein synthesis. These results further support the hypothesis that tyrosinase is necessary for the activation of the prodrug, 2,4-DOPA.

2,4-DOPA was also shown to inhibit the transport of tyrosine into MJY-alpha cells. However, since protein synthesis was not suppressed after 4-hours of incubation with 2,4-DOPA, the MJY-alpha cells apparently adapt to maintain homeostasis in the face of diminished transport.

The effect of 2,4-DOPA was also evaluated against C-1300 neuroblastoma cultures, which do not contain tyrosinase, but do contain the pteridine-dependent enzyme, tyrosine hydroxylase. Tyrosine hydroxylase is also found within catecholamine neurons in vivo. Both tyrosinase and tyrosine hydroxylase catalyze the conversion of L-p-tyrosine to L-3,4-DOPA. Therefore, the possibility existed that 2,4-DOPA might be converted to 6-hydroxy-DOPA within neuroblastoma cells, thereby producing a cytotoxic effect. If this were the case, then 2,4-DOPA may prove to be neurotoxic in vivo.

Comparison of the cytotoxicity of 5 mM 2,4-DOPA against neuroblastoma and melanoma cultures demonstrated that the melanized melanoma cells were approximately 2.9-fold more sensitive than the neuroblastoma cells. Furthermore, the treated neuroblastoma cultures were still growing rapidly, whereas the melanized melanoma cells

actually decreased in number in treated cultures. Evaluation of the dose-response of neuroblastoma cells to 2,4-DOPA indicated that 1 mM 2,4-DOPA had no significant effect upon the cultures. 2.5 mM 2,4-DOPA produced a lesser effect than 5 mM 2,4-DOPA (86 % of control and 79 % of control, respectively). The cytotoxic effect of 5 mM 2,4-DOPA could not be blocked by either AMPT or 3-iodo-tyrosine, two potent inhibitors of tyrosine hydroxylase. PTU, an inhibitor of tyrosinase, also did not block the effect. Therefore, the cytotoxicity appeared to be non-specific. Since the cytotoxicity did not appear to be mediated by tyrosine hydroxylase, catecholamine neurons may not be at risk in vivo unless they are susceptible to similar non-specific cytotoxicity. However, if the later proves to be the case when in vivo trials are initiated at a late date, a dose of 1 mM 2,4-DOPA may provide the necessary differential between neural and melanoma tissues.

In the present studies, D,L-2,4-DOPA was employed. In future studies, it may be reasonable to obtain L-2,4-DOPA for testing. It has been demonstrated (H. Rorsman, personal communication) that mammalian tyrosinase is not able to carry out the hydroxylation of D-p-tyrosine, but can only use the "L" enantiomer as substrate. It may, therefore, be the case that only L-2,4-DOPA is recognized by melanoma tyrosinase, and the "D" enantiomer contributes to non-specific (non-enzymatic) cytotoxicity. If this was the case, then the specificity of treatment might be increased by employing only the "L" enantiomer of 2,4-DOPA.

Chapter 5: SUMMARY

In summary, the experiments reported here have demonstrated that 2,4-DOPA may be of use as a prodrug in a targeted approach to the chemotherapy of malignant melanoma. 2,4-DOPA is converted by tyrosinase to the potent oxy-radical-generating toxin, 6-hydroxy-DOPA, and thereby acts to initially suppress macromolecular synthesis and to eventually cause cytolysis of melanoma cells. Doses in the range of 1-5 mM were tested, and all produced significant cytotoxicity against melanized melanoma cells. Millimolar levels of 2,4-DOPA can be obtained in vivo, and the conversion of 2,4-DOPA to 6-hydroxy-DOPA has been shown to proceed in vitro at pH's representative of the acidotic state presumed to be present in areas adjacent to the necrotic center of the tumor.

Two of the three non-tyrosinase containing cultures tested as controls (MJY-alpha mammary tumor and L-1210 leukemia) exhibited no cytotoxic effects when treated with 2,4-DOPA. C-1300 neuroblastoma cells, which were studied as a model for catecholamine neurons, exhibited 2.9-fold less cytotoxicity than melanized melanoma cells when treated with 5 mM 2,4-DOPA; 1 mM 2,4-DOPA was without significant effect against neuroblastoma. The cytotoxicity against neuroblastoma cells appeared, however, to be non-specific and could not be blocked by inhibitors of tyrosine hydroxylase. Therefore, unless catecholamine neurons are subject to similar non-specific effects, they should not be at risk during 2,4-DOPA treatment. It is hoped that these in vitro studies will serve as a useful guide for later in vivo trials of 2,4-DOPA against melanoma.

APPENDIX A: PRELIMINARY STUDIES OF THE PHARMACODYMANICS OF 2,4-DOPA IN VIVO

It was of interest to determine if mM blood levels of 2,4-DOPA could be achieved and maintained in vivo, since the K_m of 2,4-DOPA for B-16 tyrosinase was relatively high (i.e., 3.71 mM), and mM levels of 2,4-DOPA were needed for the production of cytotoxicity against melanoma cells. Furthermore, a knowledge of the half-life of the prodrug is necessary for the development of treatment schedules for future in vivo chemotherapeutic trials. To these ends, experiments were initiated in which animals were injected i.p. and the blood levels of 2,4-DOPA monitored. In some experiments, attempts were made to alter the half-life via pharmacological manipulation.

The basic procedure was as follows. After injection of 2,4-DOPA into C57BL/6J mice, blood samples were obtained by snipping a few millimeters off of the tail and gently milking out the blood. Blood was then drawn into a heparinized syringe (the syringe was dried after heparinization to avoid dilution of sample), and 20 uL was added to 160 uL of water to burst the cells. 4N PCA (20 uL) was then added to precipitate protein (note: In trial 1, Table A-1, which served as a preliminary trial, the blood was added directly to 0.4N PCA). Samples were then centrifuged and analyzed by HPLC (§2.1.2).

In a preliminary trial (Table A-1, trial 1), one mouse was injected i.p. with 1 g 2,4-DOPA/kg in a volume of 0.25 mL of 0.01 M PBS, pH 7.3. At this concentration, the injection consisted of a slurry of 2,4-DOPA which was first finely ground in an homogenization tube to avoid clogging of the needle. The blood level of 2,4-DOPA in

the mouse dropped rapidly from 1.80 mM at one hour to 0.10 mM at 3 hours (approximate half-life = 0.48 hours). It was also observed that 2,4-DOPA was decarboxylated in vivo to form 2,4-DA. The blood levels of 2,4-DA were in the uM range (Table A-1, trial 1) and appeared to decrease somewhat as 2,4-DOPA concentration decreased. The decarboxylation of 2,4-DOPA to form 2,4-DA in vivo was not surprising, since it is well-known that L-3,4-DOPA given to Parkinson patients is rapidly decarboxylated to form dopamine unless a decarboxylase inhibitor is added to the therapeutic regimen.

In the second trial (Table A-1), three mice were injected i.p. with 1 g 2,4-DOPA/kg, and the blood levels of 2,4-DOPA were monitored in order to obtain a better estimate of the half-life. As before, the the concentration of 2,4-DOPA fell rapidly from 5.29 mM ($\pm$ 0.36 S.D.) at 0.5 hours to 0.54 mM ($\pm$ 0.20 S.D.) at 2 hours. The estimated half-life was 0.454 hour ($\pm$ 0.060 S.D., N=3).

In an effort to extend the half-life to greater values, carbidopa (an inhibitor of DOPA decarboxylase) was added to the solutions injected. Mice were injected with 1 g 2,4-DOPA/kg plus either 25 mg carbidopa/kg (trial 3) or 75 mg carbidopa/kg (trial 4). The half-life of 2,4-DOPA was extended by the addition of carbidopa; both doses were equally efficacious. Addition of 25 mg carbidopa/kg extended the half-life to 0.965 hours ($\pm$ 0.045 S.D., N=3) while addition of 75 mg/kg extended the half-life to 0.929 hours ($\pm$ 0.125). In both cases, the extension of the half-life was significant with respect to control ($P < 0.001$ for both doses, SNK test).

The possibility also existed that 2,4-DOPA might be removed via

O-methylation. In order to test this hypothesis, 100 mg pyrogallol (an inhibitor of catechol O-methyl transferase)/kg was injected along with 1 g 2,4-DOPA/kg. The data of trial 5 indicate that pyrogallol had no effect upon the half-life of 2,4-DOPA (half-life = 0.454 hours ($\pm$ 0.060 S.D., N=3), $P > 0.50$, SNK test comparing pyrogallol to control). Therefore, it appears that O-methylation is not an important route of metabolism for 2,4-DOPA in vivo.

In summary, it was found that mM concentrations 2,4-DOPA could be obtained vivo. Since mM levels of 2,4-DOPA proved to be efficacious against melanoma cells in vitro, it should be possible to also obtain a therapeutic effect in vivo. However, the half-life of 2,4-DOPA appeared to be unreasonably short, even in the presence of carbidopa which extended the half-life from 0.45 to 0.95 hours. Therefore, it is recommended that 2,4-DOPA be administered in vivo via i.v. drip in order to maintain blood levels.

Table A-1

Preliminary Blood Level Studies in C57BL/6J Mice

Trial	Injection (i.p.)	Time (hours)	2,4-DOPA (mM) (S.D.)	2,4-DA (uM)
1	2,4-DOPA (1 g/kg) (1 mouse)	1.0	1.80	8.50
		2.0	0.32	5.90
		3.0	0.10	4.40
2	2,4-DOPA (1 g/kg) (3 mice)	0.5	5.29 (0.36)	
		1.0	2.50 (0.38)	
		1.5	1.20 (0.32)	
		2.0	0.54 (0.20)	
3	2,4-DOPA (1 g/kg) + Carbidopa (25 mg/kg) (3 mice)	1.0	2.35 (0.74)	
		3.0	0.47 (0.15)	
		5.0	0.13 (0.03)	
4	2,4-DOPA (1 g/kg) + Carbidopa (75 mg/kg) (3 mice)	1.0	2.56 (0.62)	
		3.0	0.38 (0.08)	
		5.0	0.12 (0.02)	
5	2,4-DOPA (1 g/kg) + Pyrogallol (100 mg/kg) (3 mice)	1.0	1.99 (0.27)	
		3.0	0.11 (0.03)	

For trials 2-5, the half-life of 2,4-DOPA was calculated by regression of ln[2,4-DOPA] versus time and was found to be (hours $\pm$ S.D., N=3): 0.454 $\pm$ 0.060 for 2,4-DOPA alone, 0.965 $\pm$ 0.045 for 2,4-DOPA plus 25 mg/kg carbidopa, 0.929 $\pm$ 0.125 for 2,4-DOPA plus 75 mg/kg carbidopa, and 0.474 $\pm$ 0.036 for 2,4-DOPA plus 100 mg/kg pyrogallol. Both doses of carbidopa were equivalent ($P > 0.5$, SNK test of trials 2-5) and extended the half-life significantly ($P < 0.001$ for both doses, SNK test). Pyrogallol did not extend the half-life of 2,4-DOPA ($P > 0.5$, SNK test).

APPENDIX B: ADJUSTMENT OF SCINTILLATION COUNTS FOR QUENCHING

The external standard method was employed to determine the efficiency of counting when transforming c.p.m. to d.p.m. Factory sealed standards (New England Nuclear) of ^{3}H and ^{14}C containing different amounts of water (quenches counts) were counted on a Hewlett-Packard model 2450 scintillation counter. The efficiency of counting was calculated from the known d.p.m. in each vial and the recorded c.p.m. The instrument also automatically determines the degree of quenching by the sample. The scintillation counter contains a high d.p.m. internal source which is counted alone and after the sample vial is placed between the source and the counter. The source is hot enough so that counts in the sample vial are negligible. The instrument then calculates the quenching due to the sample and displays the data as the A.E.S. ratio (Automatic External Standard). A plot of % efficiency versus A.E.S. ratio was then constructed (Figure B-1). The A.E.S. ratio was determined for experimental samples and used to determine the efficiency of counting.

The counting efficiency for ^{3}H in 10 mL of Liquiscint plus 0.5 mL or 0.2 mL of 1 N KOH was 37% and 39%, respectively. The counting efficiency for ^{14}C in 10 mL of Liquiscint plus 0.5 mLs of 1 N KOH was 58%.

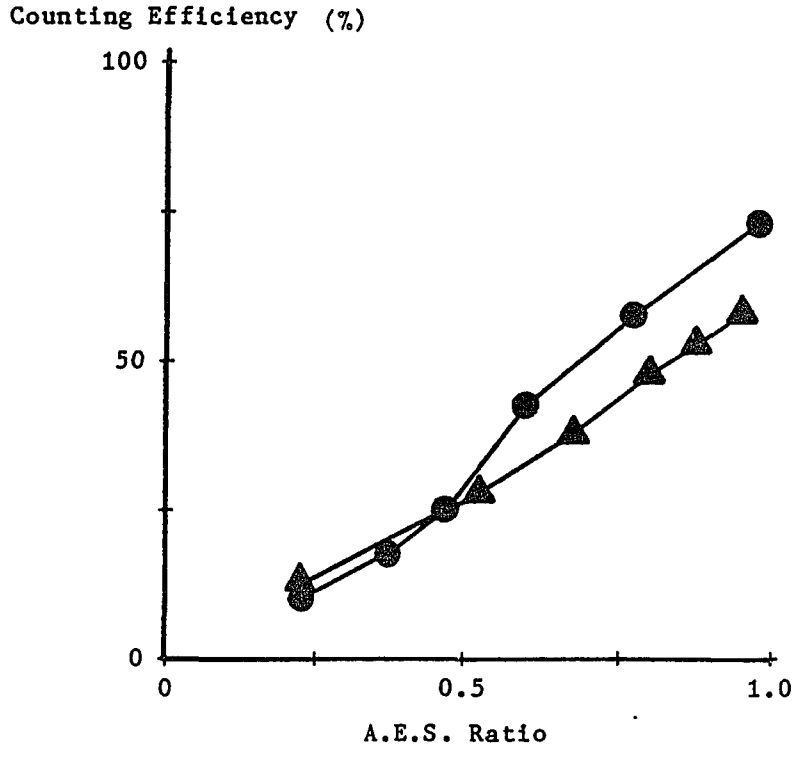


Figure B-1. Counting Efficiency as a Function of A.E.S. Ratio.

Circle = ^{14}C

Triangle = ^{3}H

APPENDIX C: CONSIDERATION OF THE POTENTIAL CYTOTOXICITY OF TYROSINASE-ACTIVATED PRODRUGS AGAINST NORMAL MELANOCYTES

The only normal cells in the body that possess tyrosinase are melanocytes. No histochemically-demonstrable tyrosinase activity is present within the mature melanosomes within the keratinocytes (Breathnach 1969). Tyrosinase activity in resting skin melanocytes is relatively low; sufficient to replace pigment that has been lost due to sloughing off of superficial layers of the epidermis. Exposure of skin to UV light, however, results in an increase in tyrosinase activity (Pavlovitch et al. 1982). Since only melanocytes contain tyrosinase and unstimulated melanocytes contain only basal levels of tyrosinase activity in comparison to pigmented melanoma tumors, many investigators have designed relatively non-toxic prodrugs which would be activated by tyrosinase to form toxic products. Use of a prodrug would target the cytotoxicity to the melanoma tumor cell and minimize systemic toxicity.

In considering the potential for a differential effect upon tumor cells versus normal melanocytes, some investigators have raised the point that "... melanocytes serve a non-essential role in the host, and therefore, potential agents need not discriminate between normal and malignant cells, ..." (Wick 1980b). However, tyrosinase activity is also present within ocular melanocytes. This deserves special consideration and is discussed below.

Dryja et al (1978) had demonstrated that bovine retina was able to catalyze the hydroxylation of approximately 0.004 nmole tyrosine/min/g tissue from 14 uM tyrosine in the presence of 230 uM L-

3,4-DOPA as cosubstrate (note: the authors did not state the counting efficiency for tritium; and therefore, an efficiency of 37% was assumed in order to convert their data from cpm/min/g tissue to nmole/min/g). In addition, the retinal pigment epithelium, iris, and choroid were also assayed; the activities were 0.14, 1.75, and 3.36 nmole tyrosine/min/g tissue, respectively.

The data of Dryja et al can be compared to data obtained by Pomerantz (1966, Figure 8) in experiments with hamster melanoma to estimate the relative difference in tyrosinase activity in the eye and in melanoma tumor tissue. Although Dryja et al. utilized non-standard assay conditions, the data of Pomerantz can be modified to account for differences in substrate and cosubstrate concentrations by taking into account the known K_m of 600 uM for tyrosine (Pomerantz 1963), $K_{activator}$ of 2 uM for L-3,4-DOPA (Pomerantz and Warner 1967), and K_I (competitive) of 400 uM for L-3,4-DOPA (Pomerantz 1966) for hamster melanoma tyrosinase. When hamster melanoma tyrosinase was incubated with 120 uM tyrosine in the presence of 120 uM L-3,4-DOPA, 3.00 nmole of tyrosine was hydroxylated/min/unit of tyrosinase (Pomerantz, 1966, Figure 8); one unit was defined as the consumption of 1 uL of oxygen/min in the presence of 0.33 mg/mL of L-3,4-DOPA at pH 6.8. Since hamster melanoma tissue contains 42.5 units/g (Pomerantz 1963), this represents the conversion of 128 nmole tyrosine/min/g tissue. Since L-3,4-DOPA was well above its $K_{activator}$ in both experiments, no correction for incomplete activation is necessary. In both experiments, tyrosine was also below the minimum concentration necessary for substrate inhibition (800 uM, Pomerantz 1966);

therefore, no correction for tyrosine inhibition is necessary. However, taking into account the difference in tyrosine and L-3,4-DOPA concentrations in Pomerantz's experiment and Dryja et al.'s experiment necessitates a correction factor of 9.1* to adjust for variation of rate with tyrosine concentration and inhibition by L-3,4-DOPA. For purposes of comparison, under the assay conditions of Dryja et al, the hamster melanoma tissue should be able to hydroxylate 14.1 nmole tyrosine/min/g tissue. Therefore, the respective tyrosinase activity of retina, retinal pigment epithelium, iris, and choroid should be approximately (< 1%), 1%, 12%, and 24% of the tyrosinase activity found within melanoma tumors. Since the levels of tyrosinase found within the retina and its pigment epithelium are 2 orders of magnitude lower than in melanoma tumor tissue, the effect of tyrosinase-targeted prodrugs upon these tissues should be minimal.

The retina should be protected from the effects of drug activation by tyrosinase within the iris, since the iris is separated from the retina by the lens, ciliary zonule, and vitreous humor. Since the anterior chamber of the eye is filled with aqueous humor which continuously baths the iris (i.e., the aqueous humor turns over approximately once every hour; Brown 1979), it is likely that any toxic metabolites generated within the iris would be cleared into the

* The correction factor = (v/V_{max}) derived from the data of Pomerantz divided by (v/V_{max}) derived from the data of Dryja et al., where:

$$(v/V_{max}) = \frac{[\text{tyrosine}]}{([\text{tyrosine}] + K_{M(\text{tyrosine})} * (1 + \frac{[\text{DOPA}]}{K_{I(\text{DOPA})}}))}$$

aqueous humor, traverse the canal of Schlemm, and enter the venous circulation to be diluted into the total body fluid.

The choroid, which also contains undesirably high levels of tyrosinase is separated from the retina by the retinal pigment epithelium, which has only trace levels of tyrosinase. Cells of the retinal pigment epithelium are joined to each other by "... tight junctions which seal the intercellular spaces between adjoining epithelial cells [and] protect the retina proper from undesirable metabolites that may be present in the stroma of the choroid" (Bloom and Fawcett 1975). Furthermore, since the choroid is highly vascular, it is likely that any toxic metabolites which may be generated within choroidal melanocytes will be rapidly cleared into the venous circulation.

From the above considerations, it should therefore be theoretically possible to achieve a differential cytotoxic effect against against melanoma cells without retinal damage as a side effect. It is hoped that this will be the case when in vivo studies are initiated at a later date. It is of interest that in the studies discussed in the Background section, no investigators using tyrosinase activated prodrugs have reported ocular damage as a side effect; however, in future in vivo studies of 2,4-DOPA, the investigators should be alerted to specifically examine microscopic sections of the eye to determine if any potential problems are present.

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