Eur J Cancer Clin Oncol, Vol. 19, No. 8, pp. 1151–1159, 1983.
Printed in Great Britain.

0277-5379/83$3.00+0.00

Estrogen Receptor in Malignant Melanoma: Fact or Artefact?

DAVID T. ZAVA and ARON GOLDHIRSCH
Ludwig Institute for Cancer Research (Bern Branch), Inselspital, 3010 Bern, Switzerland

Abstract—*A model system is presented to explain how tyrosinase, an enzyme unique to pigmented cells such as normal and malignant melanocytes can oxidize [^{3}H]-estradiol to radiolabeled products which closely resemble the tight binding of [^{3}H]-estradiol to estrogen receptor. In the model system studied, tyrosinase oxidized [2,4,6,7-^{3}H]-estradiol to [^{3}H]-water and [^{3}H]-estradiol metabolites, the latter of which formed ring-substituted conjugates with nucleophiles like monothioglycerol and BSA. Radiolabeled estradiol without tritium in the C-2 position (i.e. [6,7-^{3}H]-estradiol) failed to liberate [^{3}H]-water when exposed to tyrosinase but, nevertheless, did form ring-substituted [^{3}H]-estradiol adducts with nucleophiles. The [^{3}H]-water and the ring-substituted radiolabeled products possessed several characteristics of a genuine estrogen receptor protein in that they were resistant to dextran-coated charcoal (DCC) adsorption and their enzymatic formation was inhibited with non-steroidal estrogens like diethylstilbestrol. Other natural (estradiol) and synthetic (hydroxytamoxifen) estrogens which contain the phenol grouping also inhibited the enzymatic oxidation of [^{3}H]-estradiol. Although it was difficult to differentiate estrogen receptor from tyrosinase using the conventional DCC assay system, several differences in these two proteins permitted a distinction to be made between them. First, tyrosinase oxidation of [^{3}H]-estradiol was markedly inhibited by sulfhydryl reducing agents (monothioglycerol) that stabilize [^{3}H]-estradiol binding to estrogen receptor. Second, estrogen receptor adsorbed by hydroxylapatite whereas tyrosinase did not, thus permitting the separation of these two proteins prior to incubation with [^{3}H]-estradiol. We conclude that the [^{3}H]-estradiol binding components in melanoma previously reported to be estrogen receptor probably represent instead the radiolabeled products of the tyrosinase-catalyzed oxidation of [^{3}H]-estradiol.*

INTRODUCTION

ESTROGENS have been shown to invoke marked alterations in the growth behavior of malignant melanoma in both animal model systems [1–3] and in humans [4–7]. In humans the clinical course of melanoma often changes with the onset of puberty [4, 6], pregnancy [7] and the inception of contraceptive estrogens [5], which supports the notion that this disease is estrogen-dependent. The mechanism(s) whereby estrogens augment the growth behavior of malignant melanoma remain obscure; however, because estrogen receptor mediates the biological effects of estrogens in breast tumors [8, 9], it seems possible that malignant melanoma might also contain an estrogen receptor which modulates the action of estrogens.

Accepted 22 December 1982.

Indeed, an estradiol-binding component has been described in the soluble extracts of some melanoma biopsies [10–14]. Curiously, however, no correlation has been found between the few anecdotal cases reported of response of melanoma to antiestrogen (tamoxifen) therapy [13, 15, 16–19] and the presence of the putative tumor estrogen receptor. This suggests that the estrogen receptor in melanoma is either present but non-functional or that the [^{3}H]-estradiol binding component(s) is an artefact of the assay system used to measure this component.

One of the distinguishing features of normal and malignant melanocytes is their unique capacity to synthesize the complex polymer melanin via the enzymatic oxidation of tyrosine [20–22]. Tyrosinase catalyzes the orthohydroxylation of tyrosine to L-dopa and the consecutive oxidation of L-dopa to dopaquinone, which then

spontaneously rearranges and polymerizes to melanin (Fig. 1). Tyrosinase-catalyzed hydroxylation of monophenols consists of the insertion of one atom of molecular oxygen into the substrate ortho to the hydroxyl group already present [20, 22]. This gives rise to an orthocatechol and one water molecule. If the substrate contains tritium at the site of hydroxylation it is enzymatically abstracted from the substrate in the form of [^{3}H]-water [20]. Thus the generation of [^{3}H]-water from [3,5-^{3}H]-tyrosine has served as an index of the hydroxylase activity of tyrosinase.

Tyrosinase is not highly substrate-selective [23]. In fact, Bolt *et al.* [24, 25] observed that the A ring of [^{3}H]-estradiol is hydroxylated to 2-hydroxyestradiol, which is then consecutively oxidized to an orthoquinone. They reported that the highly electrophilic orthoquinones of the radiolabeled estradiol covalently coupled with the electron-rich sulfhydryl and amino groups of glutathione, proteins and nucleic acids, rendering the ring-substituted radiolabeled estradiol more water-soluble. Furthermore, if tritium was present in the substrate at the site of hydroxylation it was enzymatically released from the substrate in the form of [^{3}H]-water, just as [^{3}H]-water is generated from the enzymatic hydroxylation of [3,5-^{3}H]-tyrosine [20].

Because the monophenol hydroxylase and catechol oxidase activities of tyrosinase have been highly conserved throughout evolution and because these two tightly coupled enzymatic functions are essentially identical regardless of the enzyme source [22], it is not unreasonable to speculate that mammalian tyrosinase, often present in melanoma in copious amounts, might also attack and oxidize [^{3}H]-estradiol, as previously reported for tyrosinase derived from other sources [24, 25]. In this context it is interesting to speculate on how tyrosinase, present in the soluble extracts of malignant melanoma, might influence the quantification of [^{3}H]-estradiol binding components in this tumor.

In order to provide an alternative explanation for the presence of estrogen receptor in melanoma, we describe here a model system which shows that the radiolabeled oxidation products generated by the tyrosinase-catalyzed oxidation of [^{3}H]-estradiol mimic many of the properties of the binding of [^{3}H]-estradiol to a genuine estrogen receptor protein.

MATERIALS AND METHODS

Materials used in this study were purchased from Sigma, with the exception of [6,7-^{3}H]-estradiol and [2,4,6,7-^{3}H]-estradiol, which were obtained from New England Nuclear, and tamoxifen and hydroxytamoxifen (Compound E), which were kindly provided by ICI, London, U.K.

Tyrosinase (*Agaricus bisporis*) was prepared in Tris buffer, pH 7.2. Breast tumor cytosol, which had previously been identified as an estrogen receptor-positive tumor, was prepared from a frozen tumor powder in Tris buffer, pH 7.2, containing 0.0015 M EDTA, 10% glycerol and 1 mM of monothioglycerol (MTG).

Dextran-coated charcoal assay

Tyrosinase (1–2 μg/ml) or breast tumor cytosol (2 mg/ml) were incubated with [2,4,6,7-^{3}H]-estradiol (5–10 nM) or with [6,7-^{3}H]-estradiol (5–10 nM) in a total incubation volume of 200 μl in Tris buffer, pH 7.2, at 0–4°C. All assays were performed in duplicate. Where indicated in the text, tubes contained, in addition to the radioligands, a 100-fold molar excess of diethylstilbestrol (DES), 1 mg/ml BSA, 1 mM MTG or a combination of DES plus MTG or BSA. After 3 or 15 hr the incubation mixture was transferred to a pellet of dextran-coated charcoal (formed by sedimenting a 1 ml suspension of 2.5 mg Norit A charcoal, 0.025 mg dextran/ml Tris buffer, pH 8.0) to adsorb free [^{3}H]-estradiol. After 30 min at 0°C the charcoal was sedimented by centrifugation (1000 *g*). The supernatant was combined with scintillation fluid and the radioactivity determined in a Beckman scintillation counter with a counting efficiency of 30–40%.

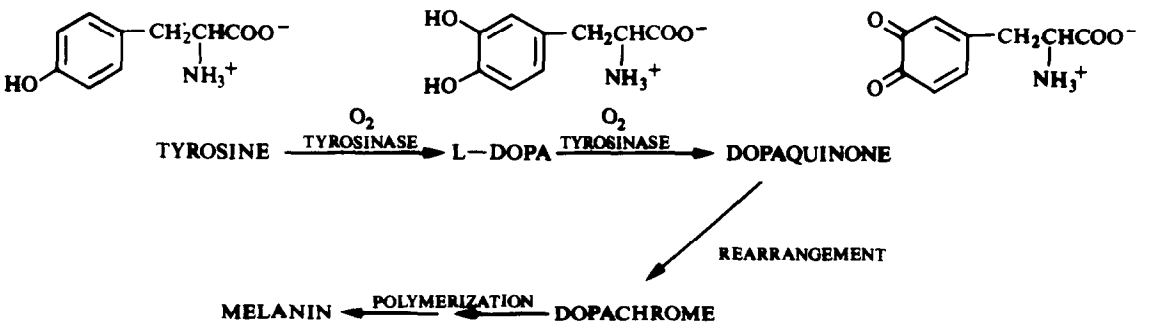

Fig. 1. Biosynthetic pathway of melanin synthesis.

Hydroxylapatite (HAP) assay

Tyrosinase and breast tumor cytosol were assayed according to the HAP method described by Garola and McGuire [26]. Two hundred microliters of tyrosinase or breast tumor cytosol were combined with 100 μl of HAP prewashed in Tris buffer, pH 7.2. The HAP was resuspended periodically and, after 30 min, sedimented by centrifugation at 300 *g* for 5 min. The supernatant was decanted. The HAP pellet was washed twice with 1 ml Tris buffer. To the washed HAP pellet was added 200 μl of 5 nM [2,4,6,7-^{3}H]-estradiol or 5 nM [6,7-^{3}H]-estradiol in the presence and in the absence of a 100-fold molar excess of DES. After a 3-hr incubation at 0°C the HAP was washed twice with 2 ml of Tris buffer. Scintillation fluid (3 ml Beckman HP) was added directly to the HAP pellet. The HAP was suspended and then poured directly into a scintillation vial. Radioactivity which partitioned into the scintillation fluid was determined after the HAP had settled in the scintillation vial.

RESULTS

Tyrosine-catalyzed liberation of [^{3}H]-H_2O *from* [^{3}H]-*estradiol*

To confirm earlier reports [24, 25] that tyrosinase generates [^{3}H]-water from [^{3}H]-estradiol when tritium is present in the substrate at the C-2 position, we compared the formation of DCC-resistant radiolabeled products following the incubation of [6,7-^{3}H]-estradiol and [2,4,6,7-^{3}H]-estradiol with tyrosinase. As a control, a breast tumor cytosol previously shown to be estrogen receptor-positive was incubated with the same two tritiated ligands. The results are presented in Table 1. As expected for a genuine estrogen receptor protein, the breast cytosol contained near-equimolar concentrations of [^{3}H]-estradiol binding sites whether the 2- or the 4-labeled [^{3}H]-estradiols were used as the binding ligand. In striking contrast, high concentrations of DCC-resistant radioactive components were generated from the incubation of the [2,4,6,7-^{3}H]-estradiol with tyrosinase which were not present when [6,7-^{3}H]-estradiol was used as the binding ligand, affirming previous reports [24, 25] that tyrosinase abstracts tritium from the C-2 position of [2,4,6,7-^{3}H]-estradiol.

Tyrosinase-catalyzed oxidation of [^{3}H]-*estradiol: enzyme inhibition by DES and heat*

Previous reports of estrogen receptor in malignant melanoma [10–13] have based results on the primary tenets that following incubation of [^{3}H]-estradiol with tumor extracts any radioactivity resistant to DCC adsorption and diminished in the presence of a non-steroidal estrogen such as DES, which has a very low affinity for serum [^{3}H]-estradiol binding proteins, is representative of a specific estradiol protein—perhaps an estrogen receptor. Further proof that the [^{3}H]-estradiol binding component is an estrogen receptor is derived from experimental evidence that the binding component is heat labile. We have shown (Table 1) that radiolabeled products from the tyrosinase-catalyzed hydroxylation/oxidation of [^{3}H]-estradiol are resistant to DCC adsorption, establishing at least one of the above-mentioned criteria of an estrogen receptor. Therefore we next determined whether the enzymatic degradation of [^{3}H]-estradiol (i.e., the formation of [^{3}H]-water from [2,4,6,7-^{3}H]-estradiol) is inhibited by DES and prior heat denaturation of the enzyme.

The results are presented in Table 2. When denatured by heat, tyrosinase was unable to catalyze the oxidative degradation of [^{3}H]-estradiol. DES markedly inhibited the formation of [^{3}H]-water by 3 hr (97%), but less so by 15 hr (47%). Total DCC-resistant radioactivity produced as a result of the enzymatic hydroxylation of [^{3}H]-estradiol was equivalent at 3 and 15 hr simulating the time-dependent saturation of estrogen receptor by [^{3}H]-estradiol. The DCC-resistant radioactivity represented slightly more than one-fourth of the total tritium label originally added to the incubation. Thus the time-dependent pseudo-saturation of the apparent [^{3}H]-estradiol binding component could be attributed to tritium being exhaustively liberated from the C-2 position of [2,4,6,7-^{3}H]-estradiol in the form of [^{3}H]-water.

Table 1. *Comparison of DCC-resistant radiolabeled products after incubation of breast tumor extracts or tyrosinase with* [^{3}H]-*estradiol*

Protein	[2,4,6,7-^{3}H]-Estradiol*	[6,7-^{3}H]-Estradiol*
Breast tumor extract†	196	225
Tyrosinase‡	973	38

*5 nM final concentrations of [2,4,6,7-^{3}H]- and [6,7-^{3}H]-estradiols.
†2 mg/ml protein concentration.
‡2 μg/ml protein concentration.

Interaction of the tyrosinase-generated oxidation products of [^{3}H]-estradiol with nucleophilic components of the incubation system

We have shown that tyrosinase is capable of liberating tritium from the C-2 position of [2,4,6,7-^{3}H]-estradiol in the form of [^{3}H]-water. However, the intermediary [4,6,7-^{3}H]-2-hydroxylated estradiol is further oxidized by tyrosinase to its respective 2,3-quinone, which according to Bolt and Kappus [24] reacts covalently with nucleophiles present in the reaction mixture. To confirm that the oxidized products of [^{3}H]-estradiol will indeed form covalent adducts with nucleophiles present in the DCC assay system described herein, a low- and a high-molecular-weight nucleophile were included in the [^{3}H]-estradiol:tyrosinase assay system. Respectively, MTG and BSA were chosen as prototype nucleophiles because the low-molecular-weight sulfhydryl reducing agents are included in the buffer systems to assess the estrogen receptor content in tumor extracts, and serum albumin is a common component of tumor extracts. In addition, the 2-labeled and 4-labeled [^{3}H]-estradiols were compared to differentiate between the formation of DCC-resistant [^{3}H]-water and the formation of [^{3}H]-estradiol adducts (measured by the resistance of [6,7-^{3}H]-estradiol:tyrosinase reaction products to DCC).

The results of Table 3 indicate that MTG influenced the oxidative degradation of [^{3}H]-estradiol by tyrosinase in two ways: first, by significantly slowing the rate that tyrosinase released [^{3}H]-water from [2,4,6,7-^{3}H]-estradiol; and second, by forming what appeared to be covalent condensation products with the oxidation products of [^{3}H]-estradiol (as measured by resistance of [6,7-^{3}H]-estradiol:tyrosinase products to DCC adsorption).

In contrast, BSA did not alter the rate of [^{3}H]-estradiol hydroxylation by tyrosinase; however, it did slightly increase the apparent radiolabeled binding components, which could not be attributed to the formation of [^{3}H]-water. This radioactive component(s) was acid-precipitable (data not shown), suggesting that the [^{3}H]-estradiol was covalently bound to the BSA. Although we did not attempt further characterization of the radiolabeled complex, we assume that it represents the formation of (a) ring-substituted thioether adduct(s), as reported previously for this reaction [24, 27, 28]. The radioactive component thus formed would be expected to acquire the physicochemical properties of the protein to which the oxidized estradiol was bound and migrate on a sucrose density gradient corresponding to the molecular weight of the protein.

Ligand specificity of inhibition of tyrosinase: [^{3}H]-estradiol hydroxylation

Information derived from the types of ligands that competitively inhibit the binding of [^{3}H]-estradiol to tumor extracts generally establish the

Table 2. Tyrosine-catalyzed oxidation of [^{3}H]-estradiol: effects of DES and heat inactivation on enzyme activity

	[2,4,6,7-^{3}H]-Estradiol*		[6,7-^{3}H]-Estradiol*	
	3 hr	15 hr	3 hr	15 hr
Control	136	143	2	1
Heat-inactivated†	9	10	2	1
+DES‡	9	75	2	1

*5 nM final concentrations of [2,4,6,7-^{3}H]- and [6,7-^{3}H]-Estradiol.
†Tyrosinase heat inactivated 90°C for 30 min.
‡100-fold molar excess of DES.

Table 3. Formation of DCE-resistant MTG- and BSA-[^{3}H]-estradiol adducts from tyrosine-catalyzed oxidation of [^{3}H]-estradiol

	[2,4,6,7-^{3}H]-Estradiol*		[6,7-^{3}H]-Estradiol*	
	3 hr	15 hr	3 hr	15 hr
Control	136	143	2	1
+BSA†	154	159	5	4
+BSA/DES‡	8	9	1	1
+MTG§	40	151	24	56
+MTG§/DES‡	17	61	15	23

*5 nM final concentrations of [2,4,6,7-^{3}H]- and [6,7-^{3}H]-estradiols.
†1 mg/ml final concentration of BSA.
‡100-fold molar excess of DES.
§1 mM final concentration of MTG.

specificity of the [^{3}H]-estradiol binding component. When [^{3}H]-estradiol binding in tumor extracts is competitively inhibited by estrogenic ligands, both steroidal and non-steroidal, the binding component is generally regarded as specific for estrogens. To explore whether a parallel exists between the ligand specificity of the inhibition of [^{3}H]-estradiol binding to estrogen receptor and the inhibition of [^{3}H]-estradiol hydroxylation by tyrosinase, we determined which types of ligands were able to inhibit these two reactions.

Figures 2 and 3 illustrate that the same panel of steroidal and non-steroidal ligands (DES, estradiol, hydroxytamoxifen) that effectively impeded the enzymatic hydroxylation of [^{3}H]-estradiol also diminished [^{3}H]-estradiol binding to estrogen receptor. Ligands containing only the aromatic (tamoxifen) or A-ring ketone grouping (progesterone, dihydrotesterone, hydrocortisone) were not very effective competitors for either reaction. Thus, with the exception of L-dopa and tyrosine, the panel of estrogenic ligands that inhibited [^{3}H]-estradiol binding to estrogen receptor were remarkably similar to those ligands that inhibited the oxidative degradation of [^{3}H]-estradiol by tyrosinase.

In contrast to the above series of ligands, L-dopa significantly augmented the enzymatic hydroxylation of [^{3}H]-estradiol without interfering with [^{3}H]-estradiol binding to estrogen receptor. L-Dopa influenced the hydroxylation reaction over

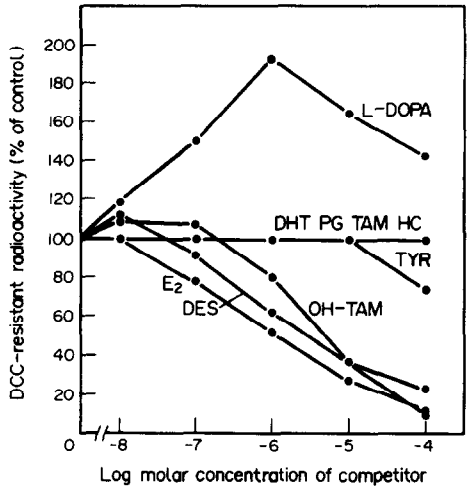

Fig. 2. Tyrosinase (2 μg/ml) was incubated with [2,4,6,7-^{3}H]-estradiol (10 nM) with or without a 1–10,000-fold molar excess of estradiol (E_2), diethylstilbestrol (DES), hydroxytamoxifen (OH-TAM), tamoxifen (TAM), dihydrotestosterone (DHT), progesterone (PG), hydrocortisone (HC), tyrosine (TYR), or L-dopa for 3 hr at 0°C. DCC-resistant radioactivity was then determined according to Materials and Methods (see dextran-coated charcoal assay). One hundred percent control value represents 2.7 pmol DCC-resistant radioactivity per μg tyrosinase.

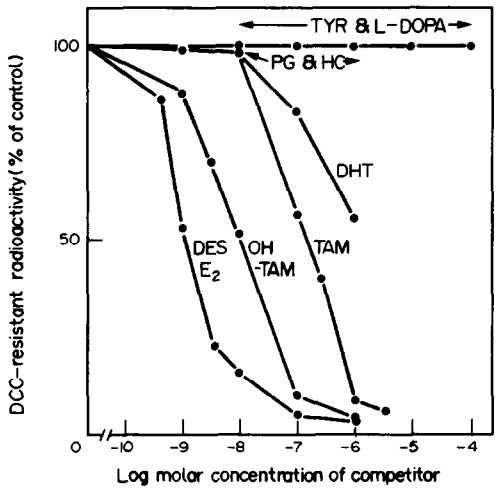

Fig. 3. An estrogen receptor-positive breast tumor cytosol (2 mg/ml) was incubated with [2,4,6,7-^{3}H]-estradiol (1 nM) with 0.5–0.1 mM concentrations of the ligands, as given in Fig. 2. One hundred percent control value represents 150 fmol DCC-resistant radioactivity per mg cytosol protein.

the entire concentration range studied (1–10,000-fold molar excess) when assessed at the 3-hr time point. These results are at variance with a previous report [14] that L-dopa inhibits the tyrosinase-catalyzed formation of DCC-resistant products from [^{3}H]-estradiol, but in accord with reports [20, 22] that L-dopa stimulates the monophenol hydroxylase function of tyrosinase (i.e. will increase the rate that tyrosinase hydroxylates monophenols). Although not shown, L-dopa did not influence the quantity of [^{3}H]-water formed at the 15-hr time point relative to control, whereas the pattern of inhibition for the other ligands was generally the same. More detailed studies in this laboratory have revealed that the difference in the effects of L-dopa at the 3- and 15-hr time points is due to differences in the rate at which tyrosinase hydroxylated [^{3}H]-estradiol. At 3 hr more [^{3}H]-water formed because L-dopa increased the early rate of hydroxylation; however, at the 15-hr time point sufficient time had elapsed to allow the exhaustive hydroxylation of [^{3}H]-estradiol with or without L-dopa.

Use of hydroxylapatite to distinguish between estrogen receptor and tyrosinase

Breast tumor estrogen receptor protein is often quantified after first being adsorbed to HAP to remove the receptor from proteolytic enzymes that would otherwise degrade the receptor [26]. The assay values by the HAP method have been shown to closely parallel the values obtained by the more routine DCC method [26]. On the other hand, Korner and Pawlek [29] reported that mammalian tyrosinase will not adsorb to HAP. Thus if estrogen receptor and tyrosinase were both present in the soluble extracts of melanoma, their

separation by selective adsorption of the estrogen receptor onto HAP would effectively eliminate tyrosinase by washing the HAP–estrogen receptor complex and should thus allow a distinction between these two proteins. To show that the HAP assay system will indeed allow a distinction between tyrosinase and estrogen receptor whereas the DCC assay system will not, we compared these two methods using tyrosinase and an estrogen receptor-positive breast tumor cytosol. The effects of using the 2-labeled or the 4-labeled [^{3}H]-estradiols as well as the effect of including MTG in these two assay systems were also assessed, since the location of the tritium in the radioligand and the presence of sulfhydryl reducing agents were shown to have profound effects on the radiolabeled products liberated by the tyrosinase-catalyzed hydroxylation/oxidation of [^{3}H]-estradiol.

Table 4 shows that near equimolar amounts of estrogen receptor could be quantified in breast cytosols by the DCC or the HAP method, by use of either the 2-labeled or 4-labeled [^{3}H]-estradiols and with and without MTG in the assay buffer.

In contrast, when tyrosinase was assayed by the DCC method high concentrations of DCC-resistant components (i.e. [^{3}H]-water) formed when the radioligand contained tritium in the C-2 position. The formation of these DCC-resistant components was further diminished by the addition of MTG to the buffer system.

When the HAP method was used to assess the interaction of tyrosinase on [^{3}H]-estradiol very little radioactivity was detectable with either the 2-labeled or 4-labeled [^{3}H]-estradiols. This apparent radioactive binding component was eliminated entirely by the addition of MTG.

DISCUSSION

In this investigation we present a model system which illuminates how tyrosinase catalyzes the oxidative degradation of [^{3}H]-estradiol to radiolabeled products that bear a striking resemblance to the products from the binding of [^{3}H]-estradiol to a genuine estrogen receptor protein when characterized by the conventional DCC assay system. The DCC assay system, used routinely to quantitate the estrogen receptor content in the soluble extracts of a variety of different tumor types, is based on the well-established principle that any radioactivity not removed from the incubation mixture by DCC adsorption and diminished in the presence of an excess of non-steroidal estrogen represents the binding of [^{3}H]-estradiol to a specific estrogen binding protein. It is assumed further that the parent [^{3}H]-estradiol is not metabolically modified during the course of the incubation.

However, as we report here, [^{3}H]-water and covalent ring-substituted adducts of [^{3}H]-estradiol are formed from the oxidative degradation of [^{3}H]-estradiol by tyrosinase (Fig. 4). Based on the DCC assay system, the oxidation products of [^{3}H]-estradiol closely mimicked the binding of [^{3}H]-estradiol to estrogen receptor in that the oxidation products were resistant to DCC adsorption, their enzymatic formation was inhibited by steroidal and non-steroidal estrogens and the covalent radiolabeled conjugates that formed from the interaction of the oxidized [^{3}H]-estradiol with nucleophiles appeared to be associated with a macromolecular component. Although we did not present evidence that the [^{3}H]-estradiol oxidation products formed from the enzymatic attack on [^{3}H]-estradiol acquire the characteristics of a high-affinity limited-capacity binder according to Scatchard [30], we did observe that if the tyrosinase concentration was maintained at less than 1 μg/ml, a sulfhydryl reducing agent (MTG) was included in the buffer and DES was used as the competitor, the radioactive products often appearing to be saturable and of high affinity (K_d = 1–10 nM). However, such results were highly variable and dependent on the intrinsic properties of the enzyme itself (i.e. the substrate turnover capacity), the length of the

Table 4. [^{3}H]-Estradiol:estrogen receptor binding and [^{3}H]-estradiol:tyrosinase oxidation assessed by DCC and HAP assays

Protein	Buffer	DCC assay		HAP assay	
		[2,4,6,7-^{3}H]-E_2	[6,7-^{3}H]-E_2	[2,4,6,7-^{3}H]-E_2	[6,7-^{3}H]-E_2
Breast tumor extract	Tris	103	86	100	77
	Tris + MTG	80	63	79	72
Tyrosinase	Tris	548	64	23	70
	Tris + MTG	428	20	0	0

An estrogen receptor-positive breast tumour extract (2 mg/ml) or tyrosinase (2 μg/ml) were incubated with 5 nM concentrations of [2,4,6,7-^{3}H]- or [6,7-^{3}H]-estradiol with or without a 100-fold excess of DES. Where indicated MGT (1 mM) was added. Breast tumor extract and tyrosinase were exposed to HAP prior to the addition of radioligands according to Materials and Methods (HAP assay). After 3 hr at 0°C DCC-resistant or HAP-bound radioactivity was determined according to the details described in Materials and Methods. Values represent fmol specific ([^{3}H]-estradiol binding – [^{3}H]-estradiol plus 100-fold excess DES) DCC-resistant radioactivity or HAP-bound radioactivity per mg breast tumor extract or per μg of tyrosinase.

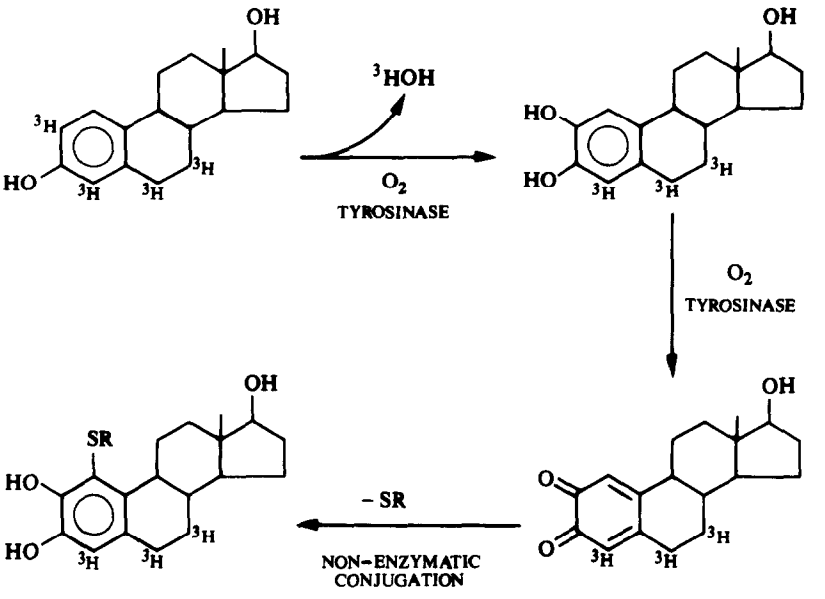

Fig. 4. Tyrosinase-catalyzed hydroxylation and oxidation of [2,4,6,7-^{3}H]-*estradiol.*

incubation period and the concentrations of MTG and DES used in the buffer system. For instance, DES was a much better competitor of the enzymatic oxidation of [^{3}H]-estradiol when MTG was added to the reaction. A dissociation constant (K_d) of 1–10 nM is about one order of magnitude higher than that reported for estrogen receptor in breast tumors [8], but is concordant with the dissociation constants reported by others [11, 14] for the [^{3}H]-estradiol binding component in malignant melanoma.

As mentioned, a [^{3}H]-estradiol binding component in the soluble extracts of malignant melanoma has been described [10–14] when either [6,7-^{3}H]-estradiol or [2,4,6,7-^{3}H]-estradiol was used as the binding ligand. Noteworthy is that in the two investigations [10, 12] using radiolabeled estradiol without tritium in the C-2 position, the incidence of the apparent [^{3}H]-estradiol binding component(s) was also lower. We observed that most of the DCC-resistant radioactivity resulting from the interaction of tyrosinase with [2,4,6,7-^{3}H]-estradiol was [^{3}H]-water, and therefore suggest that the higher [^{3}H]-estradiol binding reported in those studies using the 4-labeled estradiol might have been due partly to the formation of [^{3}H]-water. We also observed that even when [^{3}H]-estradiol did not contain tritium in the C-2 position, DCC-resistant radioactive components formed when nucleophiles such as MTG or BSA were included in the buffer system. Thus other radiolabeled estrogens without tritium at the C-2 position (i.e. [6,7-^{3}H]-estradiol, [^{3}H]-R2858 or [^{125}I]-estradiol) that might be used to assess the estrogen receptor content in melanoma extracts would not be expected to liberate [^{3}H]-water when oxidized by tyrosinase but nevertheless would be expected to form covalent adducts with available nucleophiles present in the incubation system. Such radiolabeled adducts would also be difficult to distinguish from the products of the tight binding of these radiolabled ligands to a genuine estrogen receptor protein.

McCarty *et al.* [14] observed that the radiolabeled products generated by the interaction of [^{3}H]-estradiol with extracts of melanoma or purified tyrosinase are similar in that in both cases the apparent binding components separate on sucrose density gradients as a broad band of low-molecular-weight (2–4 S) species. They speculated that the radioactive components resulted from the binding of [^{3}H]-estradiol to tyrosinase and melanosomes and to the formation of small amounts of [^{3}H]-water. We confirm that [^{3}H]-water is generated from the reaction of tyrosinase with [^{3}H]-estradiol, but only if the radioligand contains tritium at the C-2 position. Based on the experimental model presented here, showing that the electrophilic oxidation products of [^{3}H]-estradiol interact with available nucleophiles in the buffer system, we suggest further that the broad band of molecular weight components previously identified on sucrose gradients [14] probably represents ring-substituted conjugates of [^{3}H]-estradiol. MTG, albumin [24, 27] and tyrosinase itself [31], which would all be present in extracts of malignant melanoma, would be expected to interact covalently with the electrophilic oxidation products of [^{3}H]-estradiol and thus form a spectrum of apparent [^{3}H]-estradiol binding components of different molecular weight, as reported [14].

McCarty *et al.* [14] recommend that to eliminate

the oxidative degradation of [^{3}H]-estradiol by tyrosinase in melanoma extracts where tyrosinase and estrogen receptor might coexist, excess L-dopa or tyrosine should be included in the incubation system to inhibit the interaction of [^{3}H]-estradiol with tyrosinase without interfering with the binding of [^{3}H]-estradiol to estrogen receptor. This was based on the observation that neither L-dopa nor tyrosine compete with [^{3}H]-estradiol for its binding to estrogen receptor, whereas both ligands inhibit the enzymatic oxidation of [^{3}H]-estradiol. We concur that neither tyrosine nor L-dopa compete with [^{3}H]-estradiol binding to estrogen receptor. However, we observed that L-dopa but not tyrosine, even at a high molar excess (10,000-fold), did not eliminate, but rather enhanced, the tyrosinase-catalyzed hydroxylation of [^{3}H]-estradiol. It is well known that L-dopa, via its oxidation at the same catalytic site that hydroxylates monophenols, serves as an electron donor to prime the enzyme site for the reciprocal monophenol hydroxylation reaction [20, 22]. The discrepancy between our results, in which L-dopa augmented the hydroxylation of [^{3}H]-estradiol by tyrosinase (Fig. 2), and the results of McCarty *et al.* [14], in which L-dopa appeared to inhibit tyrosinase activity, can probably be explained by the differences in the manner in which L-dopa was used as a competitor. We included L-dopa as a competitor at the onset of the incubation of [^{3}H]-estradiol with tyrosinase in much the same way that other competition studies are performed. On the other hand, the aforementioned authors preincubated tyrosinase with L-dopa before adding [^{3}H]-estradiol. Preincubation of tyrosinase with L-dopa would result in the catalytic inactivation of tyrosinase via substrate turnover [20] and thereby eliminate subsequent turnover of alternate substrates such as [^{3}H]-estradiol, added later during the course of the incubation. In contrast, we included L-dopa in the incubation system with 'active' tyrosinase, which increased the enzymatic rate of [^{3}H]-estradiol hydroxylation.

Because estrogen receptor is highly thermolabile in the absence of estradiol, we would not recommend preincubating melanoma extracts with L-dopa prior to the addition of [^{3}H]-estradiol, as suggested [14]. We would also not recommend coincubation of L-dopa or tyrosine with [^{3}H]-estradiol in the assay mixture, in light of the fact that the highly reactive oxidation product (dopaquinone) of these substrates is a strong electrophile, and estradiol binding to the estrogen receptor has been reported to require an essential sulfhydryl residue [32] that might interact with the oxidation products of tyrosine or L-dopa.

We conclude that if soluble extracts of melanoma contain a genuine estrogen receptor protein in addition to tyrosinase, distinguishing between these two entities by the conventional DCC assay system may not be possible. We suggest instead that future studies on extracts of melanoma be assessed for estradiol binding components by the HAP assay system to circumvent the problem of the oxidative degradation of estradiol by tyrosinase. Furthermore, we recommend that if tritiated estradiol is used as the binding ligand it should not contain tritium in the C-2 position to eliminate the enzymatic formation of [^{3}H]-water, that the incubation period be reduced to no more than 3 hr, which allows full saturation of estrogen receptor by [^{3}H]-estradiol while reducing the enzymatic oxidation of [^{3}H]-estradiol, and that a sulfhydryl reducing agent be included in the buffer system to inhibit tyrosinase activity.

Acknowledgements—The authors are grateful to S. Saurer and B. Remy-Haldemann for technical assistance and to C. Wiedmer for manuscript preparation.

REFERENCES

1. PROCTOR JW, AUCLAIR BG, STOKOWSKI. Endocrine factors and the growth and spread of B16 melanoma. *JNCI* 1976, **57**, 1197–1198.
2. PROCTOR JW, YAMAMURA Y, GAYDOS D, MASTROMATTEO W. Further studies on endocrine factors and the growth and spread of B16 melanoma. *Oncology* 1981, **38**, 102–105.
3. KUKLA LJ, BEATTIE CW, CHAUDHURI PK *et al.* Responsiveness of porcine malignant melanoma to estradiol. *63rd Annual Meeting, Endocrine Society.* 1981, Abstract 340. Philadelphia, PA, Lippincott.
4. SADOFF L, WINKLEY J, TYSON S. Is malignant melanoma an endocrine-dependent tumor: the possible adverse effect of estrogen. *Oncology* 1973, **27**, 244–257.
5. SHAW HM, MILTON GW, FARAGO G, MCCARTHY WH. Endocrine influences on survival from malignant melanoma. *Cancer* 1978, **42**, 669–677.
6. PRATT CB, PALMER MK, THATCHER N, CROWTHER D. Malignant melanoma in children and adolescents. *Cancer* 1981, **47**, 392–397.

7. HOUGHTON AN, FLANNERY J, VIOLA MU. Malignant melanoma of the skin occurring during pregnancy. *Cancer* 1981, **48**, 407–410.
8. DESOMBRE ER, JENSEN EV. Estrophillin assays in breast cancer: quantitative features and application to the mastectomy specimen. *Cancer* 1980, **46**, 2783–2788.
9. PARIDAENS R, SYLVESTER RJ, FERRAZZI, LEGROS N, LECLERCQ G, HEUSON JC. Clinical significance of the quantitative assessment of estrogen receptors in advanced breast cancer. *Cancer* 1980, **46**, 2889–2895.
10. RÜMKE P, PERSIJN JP, KORSTEN CB. Oestrogen and androgen receptors in melanoma. *Br J Cancer* 1980, **41**, 652–656.
11. FISHER RI, NEIFELD JP, LIPPMAN ME. Oestrogen receptors in human malignant melanoma. *Lancet* 1976, **ii**, 337–338.
12. CREAGAN ET, LOPEZ RE, BHAKOO HS, ROSEN F, MOORE R, CARLSON M. Estrogen and progesterone receptors and tamoxifen in malignant melanoma. *Cancer* 1980, **46**, 1785–1786.
13. KARAKOUSIS CP, LOPEZ RE, BHAKOO HS, ROSEN F, MOORE R, CARLSON M. Estrogen and progesterone receptors and tamoxifen in malignant melanoma. *Cancer Treat Rep* 1980, **64**, 819–827.
14. MCCARTY KS, WORTMAN J, STOWERS S, LUBAHN DB, SEIGLER HF. Sex steroid receptor analysis in human melanoma. *Cancer* 1980, **46**, 1463–1470.
15. MEYSKENS FL, VOAKES JB. Tamoxifen in metastatic malignant melanoma. *Cancer Treat Rep* 1980, **64**, 171–173.
16. MIRIMANOFF RD, WAGENKNECHT L, HUNZIGER N. Long-term complete remission of malignant melanoma with tamoxifen. *Lancet*, 1981, **i**, 1368–1369.
17. NESBIT RA, WOODS RL, TATTERSALL MHN *et al.* Tamoxifen in malignant melanoma. *N Engl J Med* 1979, **22**, 1241–1242.
18. SALMON SE, MEYSKENS FL, ALBERTS DS, SOEHNLE B, YOUNG L. Tamoxifen in the treatment of malignant melanoma. *Cancer Treat Rep* 1981, **65**, 531–533.
19. FURNIVAL CM, GOUGH IR, THYNNE GSJ. Malignant melanoma: tamoxifen and oestrogen receptors. *Lancet* 1981, **ii**, 374–375.
20. POMERANTZ SH. The tyrosine hydroxylase activity of mammalian tyrosine. *J Biol Chem* 1966, **241**, 161–167.
21. SEIJI M, MIYAZAKI K. Melanization and tyrosine activity. *J Invest Dermatol* 1971, **57**. 316–322.
22. DAWSON CR, TARPLEY WB. Tyrosinase (polyphenol oxidase). In: SUMNER JB, MYRBACK K, eds. *The Enzymes*. New York, Academic Press, 1951, Vol. II.
23. PASSI S, NAZZARO-PORRO M. Molecular basis of substrate inhibitory specificity of tyrosinase: phenolic compounds. *Br J Dermatol* 1981, **104**, 659–665.
24. BOLT HM, KAPPUS H. Irreversible binding of ethynyl-estradiol metabolites to protein and nucleic acids as catalyzed by rat liver microsomes and mushroom tyrosinase. *J Steroid Biochem* 1974, **5**, 179–184.
25. BOLT HM, KAPPUS H, KASBOHRER R. Metabolism of 17-ethinylestradiol by human liver microsomes *in vitro;* aromatic hydroxylation and irreversible protein binding of metabolites. *J Clin Endocrinol* 1974, **39**, 1072–1080.
26. GAROLA RE, MCGUIRE WL. A hydroxyapatite micromethod for measuring estrogen receptor in human breast cancer. *Cancer Res* 1978, **38**, 2216–2220.
27. HELTON ED, GOLDZIEHER JW. The pharmacokinetics of ethynl estrogens — a review. *Contraception* 1977, **15**, 255–285.
28. HELTON ED, GOLDZIEHER JW. Metabolism of ethynl estrogens. *J Toxic Environ Health* 1977, **3**, 231–241.
29. KORNER A, PAWLEK J. Activation of melanoma tyrosinase by a cyclic AMP-dependent protein kinase in a cell-free system. *Nature* 1977, **267**, 444–447.
30. SCATCHARD G. The attraction of proteins for small molecules and ions. *Ann NY Acad Sci* 1949, **51**, 660–672.
31. WOODY BJB, INGRAHAM LL. Labeled tyrosinase from labeled substrate. *Nature* 1965, **205**, 291–292.
32. JENSEN EV, SUZUKI T, NUMATA M, SMITH S, DESOMBRE ER. Estrogen binding substances of target tissues. *Steroids* 1969 **13**, 417–427.