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PRESENCE AND PROPERTIES OF TYROSINASE IN SERA OF MELANOMA-BEARING ANIMALS

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Abstract. The presence and properties of tyrosinase occurring in the sera of melanoma-bearing animals were studied. Sera from mice bearing B16 melanoma were found to oxidize tyrosine and 3,4-dihydroxyphenylalanine. The tyrosinase activity in serum and melanoma tissue was inhibited by the same substrate analogues, and both had the same pH optimum. Two forms of tyrosinase could be detected by means of column chromatography using Sephadex C50. Tyrosinase activity in the serum was present in a high percentage of C57BL/6J, BALB/cJ and Swiss mice bearing B16 melanoma; BALB/cJ mice bearing Harding-Passey melanoma occasionally had detectable tyrosinase activity. Tyrosinase activity could not be detected in the serum from Syrian hamsters bearing Fortner's melanoma, not in the serum from patients with metastatic malignant melanoma.

Tyrosinase activity has been demonstrated in the serum from mice bearing B16 melanoma (5, 11). This serum tyrosinase was found to oxidize tyrosine *in vitro* under conditions similar to those required for the tyrosinase present in the melanoma tissue. It was further observed that whereas tyrosinase is mostly present in a particulate state in the tumour (8, 9), it occurs in a soluble state in the serum (5).

Presently available methods for solubilization of tyrosinase from melanoma tumour involve the use of rather drastic treatments such as treatment with acetone, butanol or detergents (1). Since the tyrosinase in the serum occurs naturally in the soluble state, it is important to study further the properties of this tyrosinase. This communication reports some properties of serum tyrosinase.

Because tyrosinase activity was consistently demonstrated from the sera of C57BL/6J mice bearing B16 melanoma, further studies were undertaken to determine whether this same phenomenon occurred in the serum of a variety of animals bearing melanomas. Particular attention was given to the question of serum tyrosinase occurring in patients with metastasic malignant melanoma.

MATERIALS AND METHODS

Chemicals

L-Tyrosine-3,5-^{3}H (spec. act. 1 000 mCi/mmole) and L-3,4-dihydroxyphenylalanine-2,5,6-^{3}H (spec. act. 1 000 mCi/mmole) were obtained from The Radiochemical Centre, Amersham, England. L-Tyrosine and naphthalene were obtained from Fisher Scientific Co., FairLawn, N. J., L-3,4-dihydroxyphenylalanine (dopa) was purchased from British Drug Houses, London, W.C.1., England, cation exchange resin AG 50W-X12 was obtained from Bio-rad. 1,4-bis (-methyl-5-phenyloxazolyl)-benzene, scintillation grade (dimethyl POPOP), and 2,5-diphenyloxazole, scintillation grade (PPO) were obtained from Packard Instrument Co. Downers Grove, Ill., Sephadex G200 and Sephadex C50 were purchased from Pharmacia, Uppsala, Sweden. All other chemicals were of reagent grade.

Tyrosinase assay

Tyrosinase activity was determined using tyrosine-3,5-^{3}H according to the method of Pomerantz (6) as modified by Menon & Haberman (5).

Dopa oxidase assay

Dopa oxidase activity was measured by determining the amount of acid-insoluble products formed from radioactive dopa. The standard incubation system consisted of the following components: 0.5 mM L-dopa, 1.0 μCi L-dopa,-^{3}H, 40 μmoles of Po_4 buffer pH 6.8, 0.5 ml of mouse serum. Total volume, 2.0 ml; temp., 37°C; incubation period, 1.0 hr. The reaction was stopped by adding 0.2 ml. 20% trichloroacetic acid (TCA) containing 1.0 mM L-dopa. The reaction mixture was kept frozen overnight. The contents were then centrifuged and the precipitate was washed 5 times with 5% TCA and then once with distilled water. The precipitate was then dissolved in 1.0 ml of 90% formic acid. 0.5 ml

Table I. *Oxidation of tyrosine and 3,4-dihydroxyphenylalanine by mouse serum*

The tyrosinase and dopa oxidase activities were determined as described under the section Materials and Methods. 0.5 ml of mouse serum was added to each tube

Serum	*n* moles of tyrosine oxidized	*p* moles of dopa oxidized
Serum from tumour-bearing mice	57	523
Serum from normal mice	Nil	35

of this solution was mixed with 10 ml of the scintillator mixture and the level of radioactivity determined. The above method is a modification of the procedure for the incorporation of tyrosine-^{14}C into melanin described by Chen & Chavin (2).

Transplantation of tumour

B16 melanoma originally obtained from Jackson Laboratories, Bar Harbor, Maine, was maintained by subcutaneous transplantation into C57 BL/6J mice every 2 weeks. The tumour tissue was mixed with 9 vol of normal saline. The tumour was dispersed by stirring with a glass rod for 2 min. Coarse tumour chunks were removed by filtration through four layers of gauze. 0.1 ml of the tumour suspension thus obtained was injected subcutaneously into the dorsal area of the neck of each mouse.

The following melanomas were also transplanted in this manner: B16 into BALB/cJ and Swiss mice, Harding Passey into BALB/cJ mice and Fortner's into Syrian hamsters. The dog melanoma employed was a spontaneous tumour.

Sucrose gradient centrifugation

Linear sucrose gradient in the range of 5%–20% was prepared using the Büchler apparatus. 0.2 ml of serum was layered over 5.0 ml of the gradient and centrifuged at 30 000 rpm for 16 hours, using the swing out rotor in an M.S.E. Superspeed 50 Centrifuge. The tubes were pierced at the bottom and the fractions were collected, each fraction consisting of 20 drops. The fractions from the three tubes were collected in the same set of test tubes, the corresponding fractions being collected in the same tubes. The tyrosinase activities of the fractions were determined as described above.

Measurement of radioactivity

The radioactivity of samples were determined by using a Packard or Nuclear Chicago liquid scintillation spectrometer. The scintillator solution used contained 50 mg dimethyl POPOP, 5.0 g PPO, and 80 g naphthalene dissolved in 1.0 l of a mixture consisting of equal volumes of toluene, dioxane and 95% ethanol. Usually 0.5 ml of the sample was mixed with 10 ml of the scintillator solution.

Reproducibility of results

All experiments were carried out in duplicate. The variation between duplicates was not more than 10%. All results presented have been corrected for nonenzymic blanks. The results are expressed as *n* moles of tyrosine oxidized per tube or *p* moles of dopa oxidized per tube.

RESULTS

Oxidation of tyrosine and dopa by mouse serum

Table I shows a comparative study of the oxidation of tyrosine and dopa by mouse serum. It should be noted that in these experiments, the experimental conditions employed to study the oxidation of tyrosine give a measure of the hydroxylation of tyrosine in the 3-position, whereas the method used in the case of dopa is such that it would provide a measure of the amount of dopa oxidized to the acid-insoluble polymer melanin. Spectrophotometric analysis of the supernatant after precipitating the acid-insoluble components did not show the presence of any intermediary compounds in the pathway of the biosynthesis of melanin from dopa. It is seen from the results given in Table I that serum from normal mice did not oxidize any detectable amount of tyrosine; but serum from melanoma-bearing mice had significant tyrosinase activity. These results are in agreement with those previously reported (5). Samples of serum from normal mice were found to oxidize small amounts

Table II. *Effect of substrate analogues on the tyrosinase activity of mouse serum*

Tyrosinase and dopa oxidase activities were determined as described in the text. The substrate analogues were added to the tubes as specified in the concentrations given below

Analogue added	Conc. of analogue added (mM)	*n* moles of tyrosine oxidized	
		Serum	Melanoma tissue
None	—	30	112
Mandelic acid	0.1	31	101
Mandelic acid	1.0	21	108
Phenylacetic acid	0.1	25	111
Phenylacetic acid	1.0	28	92
Protocatechuic acid	0.1	26	81
Protocatechuic acid	1.0	13	45
Phenylalanine	0.1	26	101
Phenylalanine	1.0	27	87
N-acetylphenylalanine	0.1	28	104
N-acetylphenylalanine	1.0	28	105

of dopa; but the amounts of dopa oxidized by sera from tumour-bearing mice were approximately 10 times as great as those oxidized by sera from normal mice. In actual practice, due to the smaller dilution factor involved in the dopa oxidase system, the dopa oxidase activity was found to be more sensitive than the tyrosine oxidase activity. Therefore, in some of the results presented in this paper, dopa oxidase activity was determined instead of tyrosinase activity.

Effects of various substrate analogues on the tyrosinase activity of mouse serum

Table II shows the effects of a number of analogues of tyrosine and dopa on the tyrosinase activity of both tumour homogenates and serum from melanoma-bearing mice. It was found that protocatechuic acid had a pronounced inhibitory effect on the enzyme activities of both tissues. Other compounds did not have any significant effect on the tyrosinase activity of either source.

Effect of hydroquinone on the tyrosinase activity of mouse serum and melanoma homogenate

A comparative study of the effects of hydroquinone on the tyrosinase activities of melanoma homogenate and mouse serum is shown in Table III. It may be seen from the results that the tyrosinase activities of both serum and melanoma homogenate were inhibited by hydroquinone almost to the same extent.

Effect of pH on the tyrosinase and dopa oxidase activities of melanoma homogenate and sera of melanoma-bearing mice

The tyrosinase activity and dopa oxidase activity of melanoma homogenate as well as mouse serum

Table III. *Effect of hydroquinone on the tyrosinase activity of mouse serum and melanoma homogenate*

Tyrosinase activity was determined as described in the text. Various amounts of hydroquinone were added to the assay system as mentioned in the table

Conc. of hydroquinone (mM)	*n* moles of tyrosine oxidized	
	Serum	Melanoma
Nil	25	67
0.1	18	47
0.5	8	25
1.0	6	5
2.0	5	0

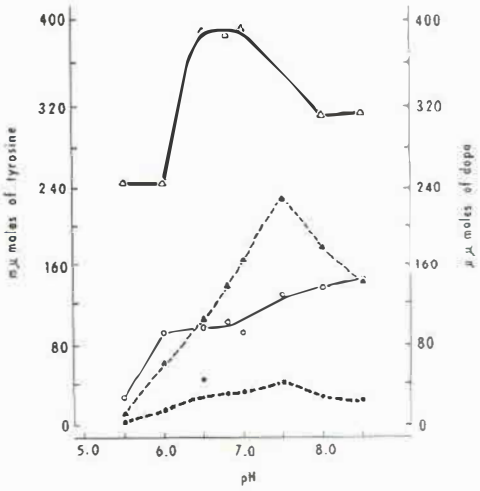

Fig. 1. Tyrosinase and dopa oxidase activities of melanoma homogenate and mouse serum at different pH values. The conditions for incubation were the same as described under the section Materials and Methods. PO_4 buffer was employed in the range 6.0 to 7.5; tris (hydroxymethyl) aminomethane-HCl buffer was employed for the pH values 7.5 to 8.5. ▲---▲, tyrosinase activity of melanoma homogenate; ●---●, tyrosinase activity of mouse serum; ○—○, dopa oxidase activity of melanoma homogenate; △—△, dopa oxidase activity of mouse serum.

at different pH values are shown in Fig. 1. PO_4 buffer was employed in the range 5.5 to 7.5 and tris (hydroxymethyl) amino-methane HCl buffer was used in the range 7.5 to 8.5. From the results at 7.5, it was noted that the PO_4 buffer or tris buffer gave the same activities for tyrosinase as well as dopa oxidase. It was found that the tyrosinase activities of both the melanoma homogenate and the serum has a pH optimum of 7.5. However, the dopa oxidase activities of the two preparations behave differently. The oxidation of dopa by mouse serum had a definite pH optimum in the range of 6.5–7.0. The activity of the melanoma homogenate in oxidizing dopa gradually increased till 8.5.

Fractionation of tyrosinase from mouse serum on Sephadex G200

Serum from mice bearing melanoma was fractionated on Sephadex G200 column. The results are shown in Fig. 2. The optical density at 280 nm as well as the tyrosinase activity of each fraction was determined. The tyrosinase activity showed a

Table IV. *Tyrosinase activities in the serum from various animals with melanomas*

Experimental conditions same as in Table I

Animal	Melanoma	Number of animals	μ moles of tyrosine oxidized		Percentage of animals having detectable tyrosinase activity
			Range	Mean	
C57 BL/6J mice	B16	72	0–372	89	83
BALB/cJ mice	B16	2	31–48	40	100
Swiss mice	B16	6	0–110	34	66
BALB/cJ mice	Harding-Passey	9	0–12	13	11
Syrian hamster	Fortner's	3	0	0	0
Dog	Spontaneous metastatic malignant	1	0	0	0
Patients	Metastatic malignant	7	2	2	0

sharp peak, coinciding with the major peak for the absorbency values.

Fractionation of tyrosinase in melanoma extract on Sephadex G200

A soluble preparation of tyrosinase was obtained by extraction of acetone-dried powder of melanoma tissue with 0.01 M PO_4 buffer, pH 6.8 (10). This extract was fractionated by using a Sephadex G200 column; and the optical density values at 280 nm and tyrosinase activities of the fractions were determined. The results are represented in Fig. 3. It was found that the tyrosinase activity was spread out in a relatively large number of fractions.

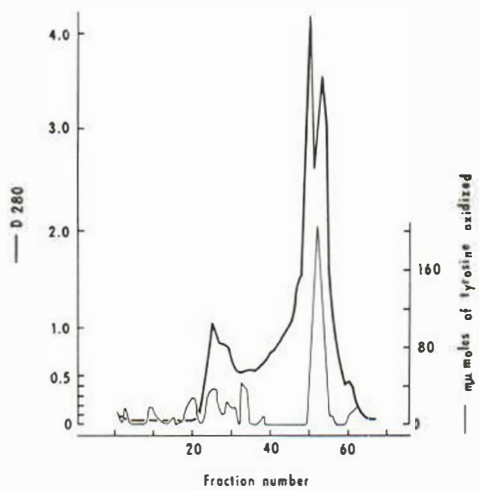

Fig. 2. Fractionation of tyrosinase in mouse serum on Sephadex G200. Sephadex column (2.5 cm × 80 cm) was equilibrated with P_{i} buffer 6.8. 3.0 ml serum was added to the top of the column and elution was carried out with the same buffer. 3.0 ml fractions were collected. The optical densities were measured at 280 nm and 1.0 ml of each fraction was employed for the tyrosinase activity.

Fractionation of dopa oxidase from mouse serum on Sephadex C50

Pooled serum from melanoma-bearing mice was fractionated on cation-exchange column Sepha-

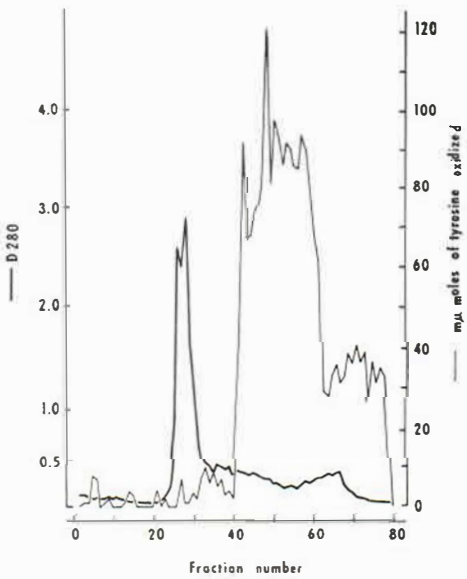

Fig. 3. Fractionation of tyrosinase in melanoma extract on Sephadex G200. Sephadex column chromatography was carried out as described under Fig. 1. Acetone powder of melanoma was prepared as described under Materials and Methods. The acetone powder was extracted by PO_4 buffer pH 6.8. 3.0 ml of the extract was added on top of the column and elution was carried out using the same buffer. The experimental conditions were otherwise same as described under Fig. 2.

dex C50. The results are shown in Fig. 4. In this experiment, the oxidation of dopa was employed instead of tyrosine because of the greater sensitivity of this system as mentioned above. It was observed that in this case the enzyme activity was present in two peaks, a major and a minor one.

Fractionation of tyrosinase in mouse serum by density gradient centrifugation

Serum from melanoma-bearing mice was fractionated by means of sucrose gradient centrifugation. The results are shown in Fig. 5. It was found that the tyrosinase activity was present only in three consecutive fractions corresponding to the central portion of the gradient.

Tyrosinase activities in the sera from various animals with melanomas

Serum samples from various animals with melanomas were assayed for tyrosinase activity. Tyrosinase activity was present in over 80% of the serum from C57 BL/6J mice containing B16 melanoma. Tyrosinase activity could also be detected in the sera of Swiss and BALB/cJ mice bearing B16 melanoma. Only 1 out of 9 BALB/cJ mice bearing Harding-Passey melanoma had detectable tyrosinase activity. Samples of serum from Syrian Hamster, dog and patients with metastatic melanoma gave no detectable tyrosinase activity.

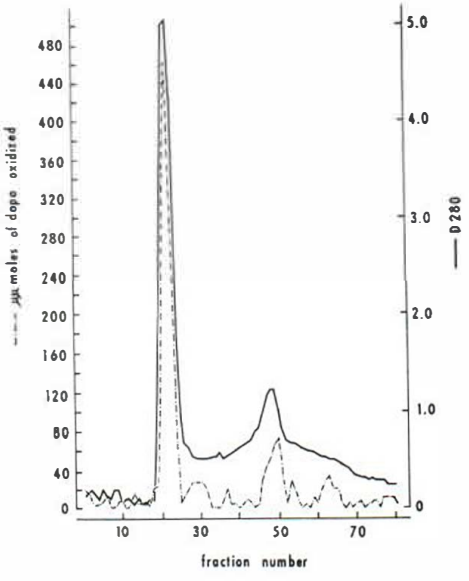

Fig. 4. Fractionation of dopa oxidase on Sephadex C50. Sephadex C50 column was equilibrated with 0.05 M PO_4 buffer pH 6.8. 3.0 ml of serum was added on top of the column and elution was carried out using a linear gradient which consisted of 0.05 PO_4 buffer pH 6.8 containing 0.0 to 1.0 M NaCl. 3.0 ml fractions were collected. Dopa oxidase activity was measured as described under Materials and Methods.

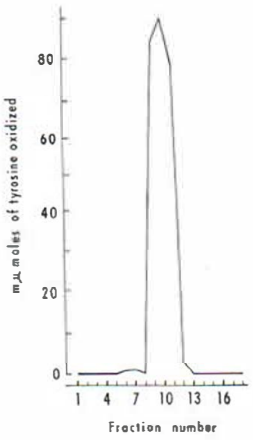

Fig. 5. Fractionation of tyrosinase in mouse serum by density gradient centrifugation. Linear gradient was prepared in the range of 5% to 20% sucrose (w/v). Sucrose gradient centrifugation was carried out as described under Materials and Methods. The tyrosinase activity of each fraction was determined by the standard system using 0.5 ml fraction.

DISCUSSION

The results presented in this paper demonstrate that serum from melanoma-bearing mice oxidize both tyrosine and dopa. Although it has not been possible under the experimental conditions to demonstrate the oxidation of tyrosine by serum from normal mice, it has been possible to show significant oxidation of dopa by serum from normal mice. There is an overwhelming amount of evidence available in the literature which indicates that the oxidation of tyrosine and dopa are catalysed by the same enzyme, viz. tyrosinase (3, 4, 7). The above results are, therefore, in agreement with this conclusion. The failure to detect tyrosinase activity in serum from normal mice while dopa oxidase activity can be detected appears to be due to the higher sensitivity of the latter system. The effects of a number of compounds structurally analogous to tyrosine and dopa on the tyrosinase activity of both the

melanoma tissue and the serum from melanoma-bearing mice were investigated. It appears from these results that both protocatechuic acid and hydroquinone inhibit the tyrosinase activity of both mouse serum and the melanoma tissue. Other compounds do not have any significant inhibitory action on the tyrosinase activity of either tissue. It appears from the above results that the tyrosinase present in the serum is similar to that present in the tumour tissue.

Studies on the tyrosinase and dopa oxidase activities of melanoma tissue homogenate and serum from melanoma-bearing mice have shown that the tyrosinase activity in both cases has a definite pH optimum, viz. 7.5. On the contrary, the variation of pH produces diverse effects on dopa oxidase activities of the tumour homogenate and the serum. The enzyme activity is the highest in the pH range 6.5–7.0 in the case of the serum, whereas the activity of the melanoma homogenate gradually rises as the pH is increased up to 8.5. The identical pH optima for tyrosinase activities in the two tissues indicate that the two enzymes are similar. However, the differences observed in the case of dopa oxidase activities of melanoma homogenate and serum do not necessarily mean that the two enzymes are really different. The oxidation of dopa to melanin involves a large number of intermediates (4, 7), and, therefore, possible additional metabolism of any of the intermediates would affect the total amounts of melanin formed. For example, dopa decarboxylase, if present in the melanoma tissue, might be expected to interfere with the melanin biosynthetic pathway. The similarity of the properties of the tyrosinase from serum and tumour presented in this paper indicates that the tumour is the source of the enzyme found in the serum. This fact is also supported by the observation that serum from white mice bearing B16 melanoma has significant tyrosinase activities. Therefore, it seems that one or more forms of tyrosinase is released from B16 melanoma tumour into the blood stream. It is possible that this soluble tyrosinase is released from the tumour before being incorporated into the melanosomes although release due to tumour necrosis cannot be ruled out.

Serum from Syrian hamster, dog and patients with metastatic melanoma failed to show detectable tyrosinase activity. Failure to detect serum tyrosinase activities in these cases could be due to one or more of the following factors: (1) Most of these tumours were not as heavily pigmented as the B16 melanoma employed. (2) The ratio of blood used for the test to total blood volume is significantly lower in the case of the larger animals and patients. (3) Inhibitors of tyrosinase have been found to be present in serum.

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